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The Regulatory Functions of Calcium and the Potential Role of Calcium in Mediating Gravitational Responses in Cells and Tissues

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Bethesda, Maryland
September 16-18, 1982

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The Regulatory Functions of Calcium and the Potential Role of Calcium in Mediating Gravitational Responses in Cells and Tissues

Edited by
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FOREWORD

In February of 1982, Dr. Thora Halstead, Chief, Space Biology Program of NASA, suggested to me that a Workshop should be held to discuss ideas and experiments on the hypothesis that calcium plays an important role in regulating cellular responses to gravity and to other environmental stimuli. In view of the preliminary information already available on the hypothesis, the timeliness of such a Workshop was evident. The proposal which I wrote to NASA for support of this Workshop was approved and funded, and in September of 1982, the Workshop was held at the FASEB Headquarters in Bethesda, Maryland.

Thirty-five scientists participated in the Workshop, all of them highly qualified as gravitational physiologists or as researchers on the cellular functions of calcium. This document is a record of the talks they gave and the ideas they shared. I believe that these talks and ideas were excellent and that this record of them will be a valuable resource for planning new experiments which will further our understanding of both cellular and gravitational physiology.

The informal, conversational language of each presentation, as printed here, reflects the fact that this volume is essentially a transcript of the Workshop rather than a formal publication. The speakers were assured that this volume would be only a transcript and many of them agreed to participate only if no formal rewrite of their oral presentation would be required. The purpose of this volume is to serve as a record of the remarkable exchange of valuable ideas which occurred at the Workshop. My wish was to get this record into the hands of the participants as soon as possible so that it could serve as a catalyst for experiments and proposals as soon as possible. The reader who expects this volume to have the polish of a formal publication will be disappointed; the reader who seeks fresh ideas, originality, and an outline of key research questions in the research areas discussed will, I expect, be very pleased.

Special thanks are due to those who helped me edit and assemble this document. The speakers themselves were the primary editors of their own talks. The task of further editing the transcript (which was superbly recorded and transcribed by Bowers Reporting Company) was shared by several researchers in my laboratory: R. Biro, B. Serlin, L. Silberman, and S. Sopory. Pat Russell at AIBS orchestrated the final printing and reproduction of the volume. I appreciated both the outstanding quality of this assistance and the generous spirit with which it was offered.

Finally, thanks again are due to Dr. Halstead. It is evident that her February suggestion was a good one.

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PROCEEDINGS

DR. ROUX: Good morning and welcome to the NASA-sponsored workshop on the Role of Calcium in Mediating Sensory Responses and the Potential Role of Calcium in Mediating Gravitational Responses. I would like to begin by thanking all of you for agreeing to participate in what I think will be a very valuable series of talks and discussions in the next few days. The workshop will be centered around a series of talks and discussions, 10 talks given by calcium function specialists and seven by gravitational physiologists.

The main goal of the workshop will be to try to evaluate whether or not calcium plays an important mediating role in some of the known gravitational responses. More specifically, we would like to address what experiments would be required to answer the question of whether calcium plays an important role in mediating some gravitational responses. Some of you may become sufficiently inspired by the discussions here to want to test your ideas experimentally. For those of you who are not familiar with the NASA research grants program there will be a handout available which will give you information on how to apply and where to apply.

What I would like to do right now, just very briefly, is to review how the workshop schedule is organized. As you are probably aware, this is a unique assemblage of people. Not only do we have gravitational physiologists meeting with calcium function specialists, probably for the first time in history but also, we have people working with animal subjects discussing ideas of research with people working with plant subjects. To try to minimize the confusion as new ideas come out, I have decided to divide up the talks into first, talks on plant systems to acquaint you with that vocabulary, and then we will proceed to the talks dealing with animal systems.

The transition between plant systems and animal systems will consist of four talks from gravitational physiologists who will review some of the phenomena that are associated with responses of plants and animals to gravity stimuli, and these talks will be independent of any information about whether calcium is involved or not. It will simply review what is known about responses of plants and animals to gravitational stimulations.

The actual formal discussions will not occur until Friday afternoon and during the morning session we have on Saturday, but I want to emphasize that I hope and expect that a lot of the really valuable discussion will occur after each talk. This is another way of saying that the speakers are going to have to allow for five to 10 minutes of discussion after their talks so that this kind of interaction can occur.

Okay, that is all I wanted to say about the schedule and the outline we are going to be following and the goals that we are going to be focusing on. I want also, today, to introduce to you a couple of people whom you will want to get to know a little bit better. The first person is Dr. Thora Halstead who is the head of the NASA Space Biology Program and administers the grants, and she wants to give you an overview of the program.

DR. HALSTEAD: First, I would like to thank you all for coming, and especially thank Stan for arranging this meeting. I think he has done an excellent job, and I am really looking forward to the discussions that will follow.

I only want to take a minute to give those of you who aren't familiar with the program an introduction to it. This workshop is sponsored by the space biology program which is a part of NASA's Life Sciences

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Division. The program focuses on gravity, both trying to understand the significance of gravity and, also, the potential of how gravity can be used as an environmental condition to probe biological questions.

The program focuses on a very broad area: the gravity sensing mechanisms both in plants and animals, the role of gravity in development and evolution, and the physiological effects of gravity, both the mechanisms involved in gravity and how gravity interacts with other environmental parameters. Although the program addresses several questions, the role of calcium in gravity-induced responses is, indeed, one of the primary questions that the program is asking.

DR. ROUX: Thank you, Thora.

The other person I want to introduce to you today is Dr. Robert Krauss who is the Executive Director of the FASEB facilities here.

DR. KRAUSS: Welcome to you all. I am delighted to see so many eager faces this morning, and I hope you have a fine workshop. If there is anything at all that we can do to make your stay more comfortable and more pleasant, please let me know. My office is over in the little stone house in the next building. I will be with you part of the time but I am afraid not all of the time. I am glad to have you here.

DR. ROUX: Thank you very much, Bob.

I will now introduce the topic of gravity effects on plants. Specifically, the topic of my talk is the possible role of calcium in mediating the principal gravitational response of plants, which is an asymmetric growth response.

The first slide is a diagramatic illustration of a plant, in this particular case a diagramatic illustration of an oat seedling which has just germinated and is beginning to grow and is showing the typical gravitational response. We call it a gravitropic response. The shoot is growing upward. The root is showing positive gravitropic response growing downward.

Gravitropism is a growth response, and as I mentioned it is an asymmetric growth response. One side is growing at a rate different from the other side. This is producing the curvature, and there are several different ways of generating an asymmetric growth response.

One would be for one side to be stimulated to grow faster than it was when the plant was growing in the upright position, and another would be for the other side to be inhibited from growing at its normal rate when the plant is in an upright position.

In fact, both of these possibilities show up as realities, but the more common situation is shown in Figure 1, and that is from Digby and Firn's work. They measured the increase in length of several different kinds of plants over time and the time zero is when the plant which is growing in a vertical position is placed in a horizontal position. This is the growth rate after gravitropic stimulation (i.e., when we place the plant in a horizontal position), and you see that the upper side decreases in its growth rate, whereas the lower side continues at the rate that it was growing before the plant was moved to the horizontal position.

Now, there are situations in some plants where, in fact, you get both responses, that is the lower side is stimulated to grow somewhat faster, and the upper side grows somewhat more slowly, but in almost all cases a common response is for the upper side in the shoot to begin growing more slowly as a result of gravitropic stimulation. So, if you wanted to focus on the cellular basis of gravitropism, or where it starts, it seems to me that an appropriate place to focus your attention would be on the the upper side. What is going on on that upper side that could possibly inhibit the growth of that upper side?

Over the years a number of different ideas have been presented on this question, and beginning in the 1960's two workers presented data which indicated that calcium accumulated asymmetrically in large amounts on the upper side of a gravitropically stimulated plant, and that this accumulation occurred before differential growth was completed. So, it could be considered one of the transduction steps important for the asymmetric growth.

Table I is taken from Goswami and Audus' work in which they looked at calcium 45 levels in bisected stems, expressed on a fresh weight basis and per dry weight basis over time, starting at time zero when the plant was first placed in a horizontal position. They compared the calcium 45 level in the upper versus the lower side. They used a plant that had been previously perfused with calcium 45 and measured what the counts per minute per milligram dry weight or per milligram fresh weight would be, and they found at time zero the distribution was relatively even, but further along in time the plant begins to curve, and by one hour, you already see the asymmetry in calcium 45 distribution.

Now, this was the third paper in a series of papers which documented the phenomenon of calcium migration following a gravitropic stimulation, and calcium accumulation preferentially on the slower growing side. Why would people focus on calcium? So what? So calcium is asymmetrically distributed; what does this have to do with growth?

It had been known, even in the sixties and certainly more information has been available in the seventies on this question, that calcium accumulation in the walls of plant cells will inhibit the growth of the cells. The mechanism by which calcium inhibits growth is not altogether clear, but it appears as though calcium interferes with some of the biochemical steps that are necessary for cell wall loosening. Plant growth occurs both as a function of cell wall loosening and as a function of turgor which then is able to extend the wall. You must have cell wall loosening in order to get growth. Apparently calcium interferes with the biochemical processes in the wall which are necessary for cell wall loosening. This was known in the sixties and known when these papers were published, and so there was a reason why these workers felt it was an interesting phenomenon that calcium would accumulate in the cells on the upper side.

Now, this work did not have very good spatial resolution. That is, all it was able to say was that calcium was on preferentially the cells in the upper half versus the cells in the lower half.

We began our investigations on this phenomenon by looking at autoradiographic studies of cross sections of oat. We found that in the coleoptile which is the sheath that covers the primary leaf and shows a gravitropic response, there is an even distribution of calcium 45 as shown by autoradiography in the unstimulated plant, and then following gravitropic stimulus, if you look in the area of the bend and do a cross sectional autoradiographic study of calcium 45 localization you see the preferential accumulation of calcium in the cells on the upper side.

This study, as well, did not have very good spatial resolution. Calcium 45 is such a high energy emitter that the kind of resolution you can get in terms of where calcium 45 is, is limited. But really in terms of a hypothesis that would say that this movement of calcium was important for the asymmetric growth, it would be necessary to say where that calcium was. Unless it was in the wall it probably wouldn't make that much difference to the growth of these plant cells.

At this time a worker in my laboratory, Bob Slocum, decided to investigate this question using a known method for subcellular calcium localization by pyroantimony precipitation of calcium in situ. This method allows visualization of dark precipitates of calcium pyroantimonate in the place or near the place where calcium was at the time the fixation was begun, and the fixation is fairly rapid and seems to occur within a few minutes. The precipitation of calcium seems to occur very rapidly.

Figure 2A is a light micrograph of the localization of calcium pyroantimonate precipitates in an upright, growing coleoptile and the dark regions that are really not all that visible, but you can see a kind of a darkening along the edges of these plants, and some of that darkening is due to calcium pyroantimonate precipitation, and you can see that there is no noticeable asymmetry of the darkening on either side of the plant. If you tilt these plants and put them at 90 degrees and then look one-half hour later at the calcium distribution or the calcium antimonate distribution you see a preferential accumulation of the precipitate in the cells on the upper side (Figure 2C). This Figure is of tissue which had been stimulated for 30 minutes. So the fixation occurred 30 minutes after placing the plants in the horizontal position, and there is barely any curvature occurring. Normally you see almost a right-angle curvature when the curvature is fully completed (Figure 2D). When the curvature is just beginning you can see already a strong accumulation of calcium in the cells on the upper side.

All this did really is verify what was already known in the literature, but verified it by a separate technique. The advantage of this technique, however, is that now you can section the same tissue regions and look at it with the EM and determine where the calcium compartmentalized.

EM graphs show dramatically that there are large calcium deposits in upper epidermal cell walls. What I can assure you is that if you look at the same cells prior to gravitropic stimulation you do not see any precipitate in the wall. Where you do see the precipitate in large quantities is in a region in an organelle in plant cells called the vacuole. It is a large central organelle that occupies often as much as 80 to 90 percent of the total volume of the cell and is known under normal conditions to have, in cases that have been studied, as high as 8 millimolar calcium inside. If you look at the unstimulated oat coleoptile cells and localize calcium with pyroantimony, you see most of the calcium localized in the vacuolar region and almost no precipitate in the wall.

Following gravitropic stimulation you see large accumulations in the wall and very little in the vacuole. If you look in the cells on the lower side where the light microscopic studies show there is very little pyroanimonate precipitate, there is no visible precipitate of pyroantimonate in the walls. So this situation is unique to the cells on the upper side, and there is a gradient where you see more in the wall as you go closer and closer to the top of the coleoptile.

SPEAKER: On the lower side do you see the antimony precipitate in the vacuoles of the cells?

DR. ROUX: Prior to stimulation, yes. Following, no. There seems to be a general clearance of calcium from the cells - on the lower side, as well as the upper side.

Now, all this is is a visual observation. It doesn't tell you what is really happening, but it suggests the possibility that part of the movement of calcium during gravitropic stimulation is a redistribution of calcium from the vacuolar regions into the wall regions. This is a plausible hypothesis. If this hypothesis is correct, then it must be true that for calcium to move from here into the cytosol into the wall there has to be the participation of calcium pumps because the normal concentration of calcium in the wall is in the millimolar range versus micromolar in the cytosol, and you have electrochemical potential difference of minus 110 millivolts inside. So, all of that would say that to get calcium out of the cell you need the participation of a plasma membrane localized calcium pump. I was aware when these studies were done that there was some information available on how this calcium pump in plant cells was controlled, and this information was provided from Dieter Marme's lab, who used crude microsomal preparations to show that calmodulin could stimulate the accumulation of calcium 45 in inside out vesicles. Unfortunately, these studies were done with crude preparations of microsomes, and so they cannot conclusively show that calmodulin is stimulating a plasma membrane localized calcium ATPase. But since Marme's studies we have done some studies on the calcium ATPase which we think is on a very pure preparation of plasma membranes and have shown basically the same response. Later on Chuck Caldwell will be speaking about his studies on calcium ATPase and the possible involvement of calcium in its stimulation.

So, this would not be too much different from the animal literature which says that calmodulin stimulates the plasma membrane localized calcium ATPase. It wasn't really a shocking result, but in terms of what I was seeing with the gravitropic response, it was an important result because it told me that if, in fact, the movement of calcium into the wall required participation of a calcium ATPase and if that calcium ATPase were controlled by calmodulin, then I might be able to inhibit gravitropism using known inhibitors of calmodulin. That was the next set of studies that I did. The first study was to show that, in fact, calmodulin occurred in the tissue that I was studying. We did isolations and showed that the material we isolated gave a strong activation of phosphodiesterase and a strong cross reactivity with calmodulin antibody. It bound to chlorpromazine and other phenothiazine drugs, and this bonding was released by EDTA. Its molecular weight was 17,000, and it, also, showed differential migration on an SDS gel depending upon whether calcium was present or EDTA was present. It had all the characteristics of calmodulin, including trimethyllysine and other known characteristics of calmodulin.

We did all these studies to convince ourselves that calmodulin was there. We, also, did radioimmunoassays and found that it was there in very large quantities: upwards of 1 percent of the total acetone precipitable protein in these oat coleoptiles is calmodulin. That is an extraordinarily large amount. So, we were encouraged to think that the hypothesis of calmodulin involvement in gravitopism was worth testing.

We began studies using chlorpromazine which blocks calmodulin's ability to activate its target enzymes. It does so by binding to the calcium activated form of calmodulin. In this case the target enzyme that I was thinking about was the plasma membrane localized calcium ATPase.

So, that was the scheme of studies. Now, I was aware and am very much aware right now that phenothiazines are very non-specific in terms of what they can do to a plant or animal cell. Especially when used at relatively high quantities, like above 10^{-5} molar, they have a number of documented non-specific effects. I was concerned about this, but I felt that I had an internal control, and that is that plant growth is very much dependent upon normal membrane functioning. A plant cannot have leaky membranes and still have normal growth rates. Anesthetics which induce membrane leakiness will inhibit plant growth. So, I felt that if I could find a phenothiazine concentration which allowed normal growth rates but still interfered with gravitropism, then the hypothesis would still be alive.

So, Ron Biro, Cal Hale and Oscar Wiegand in my laboratory began studies looking at the effects of chlorpromazine on gravitropism. The reason why we chose chlorpromazine is because C-14 chlorpromazine is available commercially, so we could investigate whether or not the drug was getting into the plant which turned out to be an important question.

When we did our initial studies we found out that when growing the plants in the presence of chlorpromazine the roots curled up tremendously, but the shoots grew fairly normally, and they showed the normal gravitropic response until we got to millimolar concentrations. We wondered whether or not chlorpromazine was even getting into the plants. It was clearly getting into the roots, but we were not sure that it was reaching the top parts of the plants.

Table 2 shows the effects of chlorpromazine on growth, not in a system where you feed the chlorpromazine through the roots, but in what I call a perfused system where the oat seedlings are being doused periodically in chlorpromazine as they move on a clinostat. A clinostat is a machine which will tumble an organism at a rate which will neutralize the net vector of gravity so that the net vector of gravity is not in any one direction. The net vector is zero, and the plant does not show any gravity response on a clinostat. We could tumble the plants through chlorpromazine on a clinostat so that it wouldn't be stimulated by gravity during the perfusion but yet it would have an opportunity to be soaked in chlorpromazine through tissue which was other than the root.

We did this because we found that the only way to get significant quantities of C-14 chlorpromazine into the target tissue, which was the coleoptile, was actually to douse the coleoptile or the top part of the seedling in chlorpromazine; feeding it through the roots simply did not work. When we perfused the coleoptiles in chlorpromazine we got a chart plotting chlorpromazine concentration versus growth of the coleoptile.

At concentrations from 10^{-11} to 10^{-6}M we get essentially normal growth rates even really in some cases supernormal growth rates. As we get above 10^{-5} molar the growth is inhibited.

So, now we felt that the concentrations of chlorpromazine to test were at or below 10^{-6}M. Could chlorpromazine inhibit gravitropism under conditions in which it was allowing normal growth rates?

SPEAKER: Are those intact coleoptiles?

DR. ROUX: The coleoptiles in Table 2 are intact, but the root is bobbed, however. The whole root is not there. We chop off the bottom part. The plant is being perfused under this situation, and it is showing these kinds of growth responses. We know using C-14 chlorpromazine that the chlorpromazine is getting in under these conditions. So, having set it up in this way, we found the effects of chlorpromazine on gravitropism in oat coleoptiles.

The way we measured the response was to look at the percent of coleoptiles which did not bend at all. We were coming across problems in how you statistically show what is going on. The problem is that when coleoptiles are treated with chlorpromazine some of them bend, but they bend with a kind of a lazy curvature that is gradual over the whole plant. How do you quantitate that kind of curvature versus the sharp curvature that normally occurs? I mean you get into some semantics as to which is more curvature than the other. So rather than get into that whole problem, we decided simply to look at the percent which did not bend at all, that were flat out following the treatment with CPZ given a 3-hour opportunity to bend. We did the test two ways in this case, tip intact or tip removed (Table 3). With the tip intact percent not bending with no CPZ was only 6 percent. As you went to 10^{-8}M, 15 percent did not bend; -7, 29%, and it really leveled out around this amount. Twenty-nine to 35 percent of the CPZ treated plants did not bend at all, significantly different from the control where you only had 6 percent.

All of these were conditions where the plant grew at normal growth rate, so you cannot say chlorpromazine was inhibiting gravitropism by inhibiting growth. It was actually in some cases promoting growth significantly over the control. Tip removal allows more chlorpromazine in, as we found using C-14 chlorpromazine. Plants thus treated showed even a more dramatic response, upwards of 57 percent not bending at all.

The next question we wanted to investigate was really a fundamental question. Is the chlorpromazine binding to the calmodulin in these plants? I mean it is nice if it gets in and so forth, but is it really affecting calmodulin at all. It turns out there is a way of covalently binding chlorpromazine to calmodulin by irradiating the treated tissue with UV light. This covalently binds calmodulin to whatever it happens to be next to. What you can do is treat these tissues with UV light for one hour and then rapidly freeze the tissue at minus 80 degrees, and then grind the tissue in acetone at minus 50 degrees to precipitate out all the calmodulin. Then you can assay the calmodulin with antibody methods to find out what percent of the calmodulin is bound to chlorpromazine. We felt that we had a really nice control in this system because about half the plants would show some curvature, and half would not show any curvature at all. We postulated that if calmodulin is making any difference at all then we ought to be able to see a difference in the amount of calmodulin that is bound up with chlorpromazine in those two situations: i.e., those that bent versus those that did not bend. We perfused plants with chlorpromazine, then separated the 50 percent of them which bent from the 50 percent not bending. We treated both with UV light and then extracted the calmodulin in both cases and assayed how many nanomoles of chlorpromazine was bound to antibody precipitable calmodulin. We made the assumption based on work in the literature that the ratio of chlorpromazine to calmodulin after binding and UV light is about one to one.

Given that assumption we calculated that there was three to four times more C-14 chlorpromazine bound to calmodulin in those plants that did not bend compared in the same experiment with those that did bend. So there is this correlation of how much chlorpromazine is bound up or how much calmodulin is tied up with chlorpromazine in the experiment where half of them bend and half of them do not bend. The hypothesis that chlorpromazine may be working through calmodulin in this particular situation is still a viable hypothesis.

It seems to me that the picture that is developing right now is that it is plausible to hypothesize that calmodulin may be involved in the sequence of events leading to gravitropic growth. If this is true, then it follows that the gravitropic stimulation, i.e., positioning of the plant in the horizontal position, somehow leads to an increase in cytosolic calcium concentration because that is how you activate calmodulin. Now, this does not explain the asymmetric distribution of calcium. It does not explain especially the major problem of how can you get asymmetric distribution external to the cell. How do you get things to move outside the cell in an asymmetric way and concentrate on one side?

That is one question. And the second question which is really more fundamental is, are the increases in calcium concentrations that we see with the antimony method sufficiently great to warrant hypothesizing that the calcium is inhibiting or playing a role in inhibiting the growth on that upper side? In other words, we can see an increase in calcium concentration but really with the antimonate method all we can say is that qualitatively there is an increase in calcium concentration. We cannot say exactly how much, and so we cannot say whether that quantity of calcium increase in the wall is sufficient and occurs sufficiently rapidly to account for the inhibition of growth that we see occurring on that side. At least it is a working hypothesis, and I would certainly appreciate input from you all as to how to go about solving some of these fundamental problems on migration of calcium external to the cell and how do you quantitate increases and how do you decide whether those increases are sufficiently great to cause inhibition.

DR. JAFFE: Stan, you say that you would expect an increase in calcium of the cytoplasm if you are activating this ATPase?

DR. ROUX: Assuming there is a movement from the vacuole to the wall, at some point there would be an increase in calcium concentration in the cytoplasm.

DR. JAFFE: Do you see that with your electron microscope studies?

DR. ROUX: No, where we pick up the story is about an hour after gravitropic stimulation and at that point we see the increase in the wall. I am not even sure that the increase in calcium concentration in the cytoplasm would be sufficiently high, sufficiently long that you would ever be able to see it with the antimonate method. You might be able to see it by another method like Roger's method where you can see a transient increase in calcium concentration in the micromolar range, because that is all it really takes to activate calmodulin. You may never be able to freeze that increase long enough to be able to see it with these standard techniques.

DR. KRETSINGER: The first question, you gave a ratio of the chlorpromazine attachment with the calmodulin to plants being three or four to one after your UV radiation.

DR. ROUX: I hope I didn't. I didn't mean to say that. I meant one to one. I thought I said one to one.

DR. KRETSINGER: No, no, the amount of calmodulin labeled--

DR. ROUX: Yes, correct.

DR. KRETSINGER: What are the absolute values? That three to one, does that three represent one part in 100; one part on 1000?

DR. ROUX: Okay, it is 11 or 12 percent versus 3 percent of the total calmodulin.

DR. KRETSINGER: 12 percent. The comment I was going to make then in any situation where there is transcellular transport, for instance, of calcium, it could be across the epithelial cells of the gut or from a vacuole at the cell surface where you have relatively too high concentrations of calcium, say, millimolar. You can quite adequately transport calcium of 2 millimolar concentrations without ever exceeding micromolar concentration in the cytosol during the process of transport, and we have done a lot of modeling studies and have looked through all the numbers. There is no necessity for the calcium concentration in the cytosol to rise very much. It must rise some, but not very much in order to affect the transport.

DR. ROUX: Do you mean to affect transport directly or to affect transport through calmodulin?

DR. KRETSINGER: Any way you want to do it. Facilitated or direct. That is not to say that it might not, but the fact that you don't detect a significant increase in cytosolic concentration doesn't preclude transcytosol transport.

DR. PICKARD: The idea of quantitating calcium concentration or increase in calcium concentration in the cell walls and inhibition of growth seems at first sight really excellent, but I think in the long run it may not be worthwhile because I don't think you can do it.

In the first place, I certainly believe the story because when we have dose response curves showing that it doesn't take very much of an increase in calcium concentration in order to inhibit growth, if you are just working with straight growth of --

DR. ROUX: Could you comment on that a little bit more because I am not aware of specifically what quantities are -- I know that in some cases as little as 2 or 3 millimolar can make a difference, but --

DR. PICKARD: Yes, I don't know. I worked with this long ago, and I don't remember numbers.

DR. EVANS: From 2 millimolar to 10 millimolar you begin to see the severe inhibition, and you get much above 10 millimolar and you are at maximum inhibition.

DR. CLELAND: It is actually lower than that if you remove the cuticle. It is down to about 1/10 millimolar that causes almost complete inhibition.

DR. ROUX: Calcium in the walls?

DR. CLELAND: Yes, well, external calcium.

DR. ROBINSON: Is there any evidence that this inhibition is acting on the wall itself?

DR. ROUX: Except that the wall doesn't grow as much, that is all. There is a correlation that there is an increase in calcium and if you increase calcium you do get inhibition. This correlation is not an absolute proof that calcium has acted directly.

DR. PICKARD: Yes, that was just a kind of prelude to what I wanted to say. It was one reason for believing that you don't need to make the measurement, but I don't think you are going to get a quantitative measurement for several reasons. One is that there is an auxin gradient there, and at concentration ranges that we know influence growth. I know you are fully aware of these data, and just generally speaking it seems very clear that the control mechanisms in all the geotropic systems are so complex and there are so many hormone interactions that you are very, very lucky and probably unreasonably lucky to get a good correlation with any one factor from side to side. In that particular system, the coleoptile, we do know that there is stimulation on the bottom, at least in many cases. So, here we have a case where you do have the calcium on the top side. I am sure it must be contributing to the inhibition, and yet we have the stimulation on the bottom side, too, and you don't see a big change in the walls. So, you know, your argument is perfectly valid without trying to make the correlations which I think are going to fail.

DR. ROUX: I still believe quantitative information would be useful if it would be possible to get it, and there may be techniques that are coming up that might be possible.

DR. ROBINSON: I just wonder about the idea of calcium, of growth inhibition by calcium acting external to the cell on the cell lives. I assume that the idea here is that the calcium is somehow or other stiffening the wall or making --

DR. ROUX: Not directly. Bob can tell you that.

DR. ROBINSON: I know there are other ideas about protons and so on involved in this, but it seems however it is working, that if you want to slow down growth, what you do is stop the processes that are involved with growth within the cell.

DR. ROUX: But one of them is cell wall loosening, and that does occur external to the cell.

DR. ROBINSON: Of course, but presumably you can have growth without inserting new materials.

DR. CLELAND: Yes, you can. There is no need to say that you have to insert anything into the wall during the growth over a short period of time, and remember this bending is occurring over short periods of time, I mean like an hour. There is no evidence of any wall synthesis going on.

DR. ROBINSON: So, it can exist just through stretching of the --

DR. CLELAND: Yes, of the pre-existing wall.

DR. ROBINSON: Okay. So, that is right to the point of what I was trying to get at.

DR. EVANS: I was intrigued by your comments that the calcium leaves the vacuole in the lower cells but doesn't appear in the cell walls in the lower cells. Do we conclude that it accumulates in the cytoplasm in the lower cells, and does this have anything to do with those tissues --

DR. ROUX: It appears as though calcium, if it leaves those cells on the lower side and gets into the apoplasm it is somehow able to move in the apoplasm to the upper side.

DR. EVANS: That is where you think it is going?

DR. ROUX: There is an actual accumulation. There is a diminution of calcium on the lower side and an increase on the upper side. This doesn't mean it is going from the lower to the upper side, but it could mean that. We don't know where it is going.

DR. KAUFMAN: I am worried, Stan, about your kinetics for the generation of curvature, the initiation; you said 30 to 45 minutes, and I worked with Bob's corn coleoptile on our transducers. They start within five minutes. I don't know of any start within 20, and I am sure your oat coleoptiles must start much earlier, and so your asymmetry is established way after initiation of curvature.

DR. ROUX: Okay, we can get the coleoptiles to curve faster. There are ways of doing that, but when we were doing these experiments, visibly any curvature that we could see at all did not occur until about 40 minutes.

DR. KAUFMAN: You didn't put them on a transducer, did you?

DR. ROUX: No.

DR. KAUFMAN: Because you would get the fine kinetics, very excellent resolution and begin to see that probably within five minutes.

DR. ROUX: Okay, but now the calcium asymmetry begins within 10 minutes.

DR. KAUFMAN: Still after the initiation of curvature.

DR. ROUX: If curvature in these plants occurred within 10 minutes, and I am not sure that they did.

DR. KAUFMAN: You need a sensitive angular transducer to nail that down.

DR. ROUX: Yes, and I would like to talk to you about that.

DR. TSIEN: It seems to me that a crucial element of your hypothesis is that there will be a calcium gradient in the extracellular medium between the bottom and the upper phase. Has anyone looked into that? It would seem to be straightforward enough to look for with extracellular calcium electrodes, something like 1 millimolar or even tenths of millimolar. It is not very high, of course, in the best sensitivity range for a calcium electrode, and then we could see whether or not the trends are through the whole plant.

DR. ROUX: The calcium electrodes that I am familiar with are rather large.

DR. TSIEN: The state of the art now is that you can stick them into 10 micron animal cells. So, maybe it is the key intracellularly. I don't know what plant walls would get in the way, but I would have thought you could get a 20 micron or a 10 micron one into the interstices of cells and look for the actual gradient. If you could measure it, then you could start to mill it by putting calcium gradients in your plants and just seeing whether or not auxin and what is left actually removes calcium.

DR. BANDURSKI: In a complex in vivo system it is relatively meaningless because the wall is loaded with polyuridines and so on which will bind calcium very, very effectively. What you really want to know is how much calcium is in the presence of Don Nivens' enzyme, you know, the one where you can inhibit with antibodies.

Now, the real question is how much calcium does that --

DR. ROUX: How much that enzyme needs to be inhibited?

DR. BANDURSKI: Yes, I mean that is the concentration gradient, and you are not at all interested in the calcium content of the wall, but rather calcium content with regard to that enzyme. People who don't work with enzymology sometimes forget that the whole organism sees one thing, but the enzyme sees a totally different thing

DR. ROUX: That is a very good point.

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ROUX TABLES

TABLE 1. *Effects of horizontal exposure on ^{45}Ca distribution in hypocotyls of* Helianthus annuus *seedlings. Means of four replicates*

Time in horizontal position (h)	Side	^{45}Ca levels ct/min mg d. wt	 ct/min 10 mg f. wt	Curvature (means ± S.E. mean)
0	Upper	56·05	24·07	
	Lower	54·12	22·90	
1	Upper	49·40	22·02	5.1 ± 0·25
	Lower	35·85	14·42	
2	Upper	64·17	25·92	35·1 ± 1·50
	Lower	42·30	16·37	
3	Upper	87·92	35·15	70·2 ± 2·19
	Lower	48·40	19·30	

from: Goswami, K. & L. Audus. 1976. Ann. Bot. 40: 49-64.

TABLE 2. *The effect of exogenous CPZ on extension growth of oat coleoptiles with intact tips*

Group	CPZ (M)	Change in length (mm)
A	0	6·1*a*
B	10^{-7}	5·9*a*
C	10^{-6}	7·1
D	10^{-5}	7·7
E	10^{-4}	4·0

All plants were grown in nutrient solution to 27 mm before CPZ treatment. Final measurements were taken 8 h after initiation of the perfusion treatment. Each value is the average of at least ten measurements. Values followed by the letter *a* are insignificantly different from one another by *t*-test at the 95 per cent confidence level. All other values were significantly different from one another.

from: Biro et al. 1982. Ann. Bot. 52, 735-747.

TABLE 3. *The effect of CPZ on gravitropism in oat coleoptiles*

Treatment	CPZ (M)	Percentage not bending
	Through roots only	
Whole plants	0	0*a*
Whole plants	10^{-7}	0*a*
Whole plants	10^{-6}	3*a*
Whole plants	10^{-5}	5*a*
Whole plants	10^{-4}	5*a*
	Perfused	
Tip intact	0	6*a*
Tip intact	10^{-8}	15
Tip intact	10^{-7}	29
Tip intact	10^{-6}	27
Tip intact	10^{-5}	35
'Decapitated'	0	10*a* (7·0 ± 0·9)*
'Decapitated'	10^{-6}	38 (5·5 ± 2·1)
'Decapitated'	5×10^{-6}	66 (5·0 ± 0·8)

* Indicates the average bending height of those coleoptiles which did bend ± standard deviation.

Whole plants were exposed to CPZ at their roots only for 6 h in the growth chambers. Perfused plants were transferred to perfusion clinostats for CPZ treatment. Coleoptiles with their apical 1–2 mm tip removed ('decapitated') were supplemented with 6×10^{-6} M IAA during perfusion. The perfusion period was 6 h for tip intact coleoptiles and was 12 h for 'decapitated' coleoptiles. All observations were made 3 h after gravitational reorientation. Values followed by the letter *a* are insignificantly different by *t*-test at the 95 per cent confidence level.

from: Biro et al. 1982. Ann. Bot. 51: 735-747.

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ROUX FIGURES

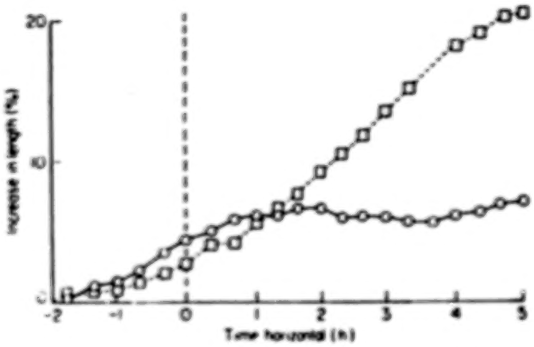
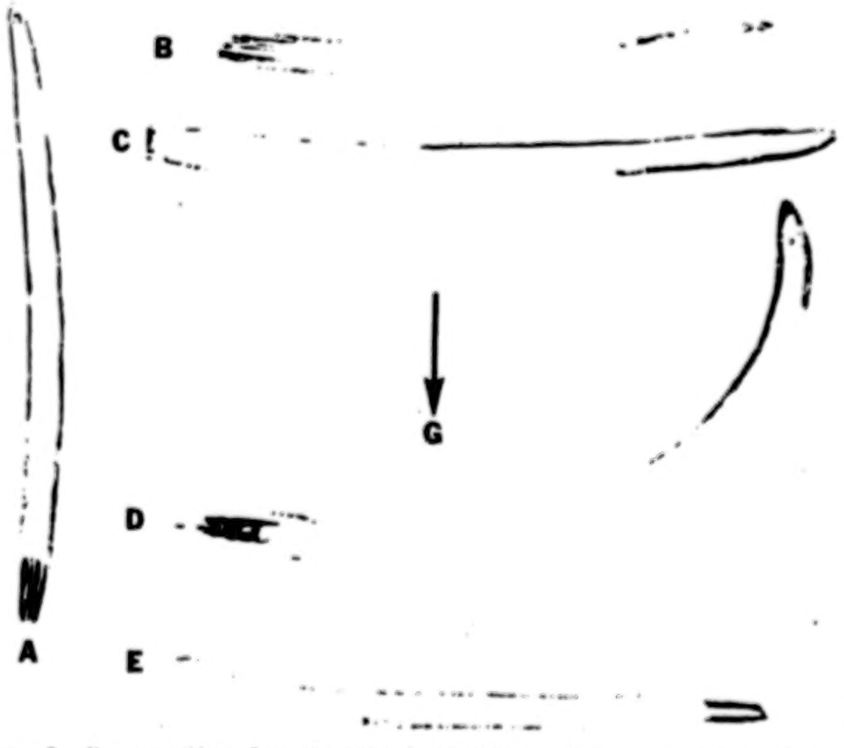


Figure 1. Growth of the two sides of an etiolated seedling of *Zea mays* (5 days old) before and after geostimulation. The seedling was orientated vertically for 2 h and then held horizontally for a further 5 h. The coleoptile and the upper part of the mesocotyl were measured. ○—○ Growth of upper side, □- - -□ growth of lower side. from: Digby, J. and R. Firn. 1979. Plant, Cell and Environ. 2: 145-148.



Note: Letters are placed at the base of all sections, except for "E".

Fig. 2. Near median longitudinal sections through antimonate-stained oat coleoptiles, before and after gravistimulation. A) unstimulated, upright coleoptile. B) coleoptile after 10 min. gravistimulation, C) 30 min. gravistimulation, D) 3 hr. gravistimulation. Fig. E shows a coleoptile which was pre-treated with 10^{-6}M CPZ for 6 hr. prior to 3 hr. gravistimulation. Note lack of curvature and lack of calcium precipitate asymmetry (dark areas). Blackened areas near cut ends of coleoptiles show wound response to cutting: phenol accumulation. This Figure is taken from the Ph. D. thesis of R. D. Slocum (1981).

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DR. ROUX: I would now like to introduce the next speaker, who comes to us from the University of Massachusetts, Peter Hepler, whose topic will be calcium and mitosis.

DR. HEPLER: I must say I feel very awkward being labeled as a calcium expert. I am really quite ignorant about calcium. I would like to consider myself more of a calcium enthusiast, and I guess like others I am here, hopefully, to learn about calcium. But for the next 35 or 40 minutes, I would like to tell you about some of the things that we are thinking with regard to calcium and specifically calcium and mitosis.

Mitosis is common to all eukaryotic organisms. The mitotic apparatus has much the same structure throughout plants and animals to the extent that it is composed of microtubular spindle fibers, etc. Thus the process we will focus on here is common and fundamental to every living organism. The hyperbole that I use on the role of calcium and mitosis is that if you ask the person on the street what regulates mitosis, he/she will say, of course it is calcium. The reason for this I think is obvious. Ten years ago this month Richard Weisenberg showed conclusively that microtubules are sensitive to varying concentrations of calcium; as you raise the concentration of calcium, microtubules depolymerize. This was the key that allowed the biochemists to obtain in vitro polymerization of microtubules.

A second calcium regulated process might be microtubule dynein activation. We now are becoming quite certain that dynein, the cross bridge between microtubules in cilia and flagella, is a calcium-regulated protein. If microtubule sliding occurs in the mitotic apparatus then we imagine that it would be driven by a dynein ATPase (1).

A third calcium regulated process could be activation of a microfilament system. A small but vocal group of people say that actin microfilaments are the motor for mitosis. If actin is involved you would expect it to be calcium regulated, probably by a calcium, calmodulin stimulated phosphorylation of a myosin light chain that would allow myosin and actin to bind and stimulate the myosin ATPase activity.

A fourth process might be gel-sol transformations which would also involve, presumably, an actin filament network. This derives its strength from some recent studies of Taylor and Condeelis (2) working on cell-free extracts of Dictyostelium where they are able to generate a gel in vitro. Adding calcium stimulates two events; firstly, a solation of the gel, and secondly, a contraction of the gel network. The mechanism by which this acts may be by some calcium sensitive actin-binding protein that is perhaps clipping or breaking the actin filaments and allowing solation and then subsequent contraction to take place.

The above processes are just four general ways of thinking about how calcium might interact with the mitotic apparatus and affect the motion of chromosomes. There may be many others. In fact, calcium could be imagined as working on all of these, that is to say two or more might be operating together. Thus it can be imagined that an increase in calcium concentration could stimulate two or three of these which could work cooperatively or even synergistically to move chromosomes (3).

While we can develop ideas on how calcium can affect mitosis, what evidence do we have that it actually plays a role? That I would like to address, and from the literature we can generate some arguments. For example, we already know from a general point of view that an increase in calcium may stimulate development that leads to a mitotic and meiotic event. A good example is found in the starfish oöcyte which is arrested in miotic prophase. A hormone, 1-methyladenine, causes an increase in calcium and then the nuclear envelope breaks down and the cell proceeds through meiosis.

Recently a student of mine, Mary Jane Saunders, has correlated an increase in calcium with development and mitosis in kinin-induced bud formation in the moss Funaria. She has shown that with the ionophore A23187 one can introduce a calcium increase that stimulates bud formation in the absence of cytokinin. We can start to understand the fact that calcium is an important mediator of development, somewhere in the chain of events leading to a developmental event that involves mitosis or a series of mitoses.

But what about the mitotic apparatus itself. In what I have just reported, calcium could actually be acting at some point distant from the mitotic apparatus, the microtubules and microfilaments. Now, I would like to briefly summarize published evidence that shows the possible involvement of calcium with the mitotic apparatus.

If calcium is injected into a cell, e.g. sea urchins, it will cause a decay of the spindle birefringence that is presumably due to a breakdown of the microtubules (4). One of the interesting sidelines here is that that birefringence decay quickly vanishes and the spindle regrows, suggesting that the calcium that has been introduced is very rapidly resequestered. In isolated mitotic apparatuses increasing calcium also causes a depolymerization of the microtubules (5).

More recently Cande (6) has shown that calcium in the .1 to .5 micromolar range will stimulate anaphase motion of chromosomes. These are permeabilized mammalian cells, and as the concentration of calcium increases the chromosomes move more rapidly to the poles.

In addition, there is evidence for calcium ATPases in the spindle, and that these ATPases fluctuate in activity in correspondence to the events of mitosis. There is, also, evidence that mitotic apparatuses that are isolated mechanically so that their membranes are intact will sequester calcium. Finally I mention that calmodulin is localized in the mitotic apparatus of dividing cells. Calmodulin seems to be localized in the polar regions of the cell, and some very elegant work of DeMey and Coworkers (7) shows that the polar ends of the microtubules seem to be encrusted with calmodulin.

Thus, we are obtaining evidence about a calcium role in mitosis; yet we are far from understanding the mechanism of calcium regulation, compared to how the muscle physiologist understands the role of calcium in muscle action. It is at this point that I became very interested in the problem. I would like to tell you about some of the experiments that we have been doing. The various topics I want to cover briefly are: firstly, that the spindle apparatus contains a highly developed membrane system that has many characteristics of the sarcoplasmic reticulum of muscle; secondly, that this membrane system contains calcium; thirdly, that there are ionic fluxes occurring during mitosis which we can see by a variety of fluorescent probes; and, finally, very briefly address the issue of whether through modulation of the calcium experimentally we can modulate the process of mitosis and thereby better understand it.

Figure 1 shows leaf cells of barley. This happens to be a guard mother cell. What I have done here is to borrow a technique from muscle ultrastructural physiologists who have developed what is called the osmium ferrocyanide postfixation technique to stain sarcoplasmic reticulum of cardiac muscle (8). Notice that it clearly stains the nuclear envelope and endoplasmic reticulum of the cells and permits one now to see with much greater clarity than heretofore the particular distribution and structure of these membranes (3,9). It turns out that the bulk of membranes in the mitotic apparatus are smooth and tubular, and the conventional glutaraldehyde osmium techniques are simply not adequate to bring out these kinds of membrane conformations, a fact that is not generally realized. For example Fuge, in his review on the mitotic apparatus says that membranes are not a very common or important part of the mitotic apparatus. His conclusions are based on pictures taken with the conventional preparation which I submit do not show the membranes clearly.

In Figure 2 we have the metaphase plate. We have some membranes coming into the mitotic apparatus, and we have membranes virtually surrounding the mitotic apparatus.

SPEAKER: What is the stain again?

DR. HEPLER: It is a mixture of osmium tetraoxide and potassium ferricyanide and the mechanism by which it is working is not clear, but it may be linking to various metalloproteins on membrane surfaces and causing a precocious dumping of cyano-bridged iron osmium complexes, but the chemistry is not all that well understood.

In prometaphase, the nuclear envelope has just broken down. One of the first membrane differentiations one sees is an accumulation in the spindle pole. At higher magnification one sees a plexus of smooth, tubular endoplasmic reticulum at the spindle pole, between which are a number of short segments of microtubules. This is the microtubule organizing center of the dividing cell.

DR. KEEFE: Are those all single isolated tubular profiles?

DR. HEPLER: No, they are all connected. Serial section shows that this is fully connected. If you put a tweezer on this membrane you can pull the whole thing out. An example of a cell in metaphase shows that even though the nuclear envelope has broken down, the spindle is still virtually surrounded by an intact membrane system. Elegant work by Kubai on monopolar meiosis in Sciara, a Dipteran fly, shows through serial section reconstruction, that a membrane sheath entirely surrounds the spindle apparatus. Thus, this is not particular to lettuce or barley and not even particular to plants.

One of the other things that I think is very exciting and that is shown in Figure 2 is that there are intrusions of membranes into the spindle, and these intrusions occur specifically along the kinetochore bundles. There are accumulations of membranes in the spindle pole, and in structure they appear sometimes as a tubular reticulum, and sometimes as a lamellar membrane which has fenestrations in it. You might be interested in the observation that these fenestrated elements look quite similar to the fenestrated collar of the muscle sarcoplasmic reticulum where it overlies the A band. The tubular network is not unlike the tubular reticulum that connects the fenestrated collar to the terminal cisternae of muscle SR. Thus, there are a number of structural similarities between this mitotic membrane system and muscle SR (9). In muscle SR of course, is the calcium-regulating system.

Now I return to the images showing the membrane invasion into the spindle apparatus, specifically along the kinetochore fibers (Figure 2). Through serial section reconstruction you can show this is one fully interconnected system. That this is not just a product of osmium ferrocyanide is shown in material fixed with conventional glutaraldehyde.

I had no idea this was going to be such a botanical meeting. Almost always I feel I have to justify these results to the audiences I am talking to. Recently I have been spending my summers at Wood's Hole, and one does not have a real phenomenon until you show it in sea urchins. An examination of membranes in Arbacia, the common sea urchin at Wood's Hole, shows that it is virtually packed with membranes. This is already known from the work of Harris (10), but I think it helps to keep emphasizing it. At the spindle pole is a centriole. It has a halo of surrounding material and tubules going every which way. Between the microtubules the spindle is packed with membranes. In fact, I think that membranes make up a greater percentage of the spindle mass than the microtubules.

Sue Wick, in my laboratory, has used the potassium pyroantimonate procedure to localize Ca^{2+} deposits in dividing cells (11). Examining cells of the stomatal complex and spermatogenous cells of Marsilea where we know from the previous electron micrographs that the membranes are very much enriched in the polar regions and not so many in the interzone, she sees numerous black precipitates in the membrane area. We believe these to be calcium antimonate; by and large they are localized in cisternal spaces of the endoplasmic reticulum. You do see precipitates in the mitochondria, plastids, golgi bodies, the vacuole and the cell wall. The deposits are thought to be calcium antimonate as judged by a series of controls that Sue Wick did, using chelators to remove the stain; EGTA and EDTA are strong dissolvers of these black deposits. Using known precipitates of potassium, magnesium and calcium antimonate, Sue embedded them and treated them like material. She saw that in those chemically prepared preparations, it was the calcium antimonate that behaved like the deposits on the tissue. In another example, again a subsidiary cell of barley leaf, these deposits are highly clustered in the membrane rich area of the spindle pole. Within the ER the deposits occur in the cisternal spaces. We feel from these observations that the membrane system is rich in calcium.

We have, also, approached the question from another point of view. Steve Wolniak, a former postdoc with me, now at the University of Maryland, used chlortetracycline, a fluorescent probe that appears to indicate membrane associated calcium (12, 13). The tissue that he studied was the endosperm of Haemanthus, the African blood lily which is a very elegant object for light microscopy. A cell in late prophase shows the nuclear envelope faintly outlined. One also sees, at this stage that the spindle region itself has somewhat brighter chlorotetracycline fluorescence. One furthermore, sees that there are patches of fluorescence that probably are due to aggregations of organelles like mitochondria and plastids in the cytoplasm. Thus, during prophase, the polar region itself becomes quite bright. In metaphase, the fluorescence chlortetracycline or what we presume to be membrane-associated calcium signal is similar to that of calmodulin localization in a variety of mammalian cells.

A highly flattened metaphase cell of Haemanthus (Figure 3), contains a region of the spindle which is pretty much devoid of chromosomal arms. Within this region we can start to pick up a differentiation of the fluorescence. We see these little wisps or cone-shaped arrays of fluorescence that extend into the spindle, and they point exactly to the kinetochores. Those are the light regions on the chromosomes.

DR. LEOPOLD: Beautiful pictures. How do you get the same cell with your fluorescence shot and the fixation?

DR. HEPLER: The cell has been treated, and we just move very quickly. We have a Reichert microscope on which we can do phase, fluorescence and polarization.

Thank you.

DR. PICKARD: Is that reflected light rather than transmitted?

DR. HEPLER: No, this is incident light fluorescence. The phase, of course, is transmitted, and the polarization is transmitted, too.

A second highly flattened cell of Haemanthus shows that the fluorescent wisps, corresponding to calcium rich membranes overlie exactly to the birefringent spindle fibers.

I don't have a slide, but Steve has used a detergent to remove membrane, but retain the spindle microtubules. In other words you still see birefringence, but under conditions in which the membrane has been removed there is absolutely no fluorescence. We interpret this as indicating that the chlortetracycline is labeling a membrane, a subset of membranes which are specifically associated with the kinetochore fibers in those particular regions.

Figure 4 is a cartoon which briefly gives a model of how I see the dividing cell at this point. Membrane rich area in the poles, and membranes extending along kinetochore fibers. Perhaps by some triggering mechanism calcium could be released, say, at the metaphase/anaphase transition and stimulate a variety of processes that I mentioned at the very beginning of our talk, such as microtubule depolymerization etc., to cause chromosomes to move to the poles.

What kind of evidence do we have that these ions are changing? The first evidence is from the work of Steve Wolniak. What he did was to take these same cells that we have been looking at, treated with chlortetracycline and use a sensitive photomultiplier tube to detect changes in fluorescence over localized regions of the spindle (14). Using a 12-micron spot, he looks over a clear region of the spindle between the chromosomes and the poles, and he watches for changes in fluorescence. The onset of anaphase is indicated by a vertical line in Figure 5. The lower graph is plotting out the movement of chromosomes to the poles, and the upper graph plots, in relative units, changes in fluorescence of chlortetracycline (Figure 5).

Normally, in chlortetracycline, some five to 10 minutes before the onset of anaphase there is a sharp reduction in chlortetracycline fluorescence, that usually remains level as the chromosomes are moving to the poles. In two of seven cells we have analyzed the fluorescence has gone up again during later anaphase. These results are consistent with an efflux of calcium from the membrane system. There have been several studies now that have shown both in vitro and in vivo that a reduction in fluorescence does indicate a release of calcium from membrane systems in muscle, in platelets and so forth.

An important control in my view is to test whether those changes are just due to a topology or distributional change in the membrane. We used a general membrane stain N-phenylnaphthalamine (Figure 6). Except for a little dip right here as the cell starts to pop into anaphase, the signal remains essentially constant, arguing that the changes we see with chlortetracycline are not due simply to a structural redistribution of the membranes (14).

DR. BIKLE: You measure the fluorescent signal. Are you measuring that over the whole cell?

DR. HEPLER: No, we are measuring it over a spot that is over the spindle, but we can move the spot around. It is a 12-micron spot, and these spindles are huge. They are in the neighborhood of 50 to 100 microns in diameter.

DR. BIKLE: The presumption I would have is that if you put it at the pole you would not see any change.

DR. HEPLER: Yes, we will get to that. We have not done that so much with chlortetracycline as we had with two voltage sensitive dyes, ANS- and DiOC$_5$(8). Using these two so-called "voltage sensitive" dyes, DiOC$_5$ and ANS, Steve Wolniak has examined fluorescence changes in (S), the spindle region; (P), the polar region; (N), the non-spindle region, that is the cytoplasm totally outside of the poles and the spindle (Figure 7 (14)). Again, the lower graph displays the motion of chromosomes to the poles. With DiOC$_5$ and ANS we get a sharp increase in fluorescence only in the spindle region. We do not see it in the pole or non-spindle cytoplasm. In fact here in this particular cell, the non-spindle cytoplasm may have even dropped (Figure 8).

DR. ROBINSON: Are you trying to say that these are reflecting a membrane potential change in this membrane system we are looking at?

DR. HEPLER: Okay, there is some kind of change in ions, I hesitate to say it is membrane potential. We don't know enough about these dyes or the system. What I think we can say is as follows, that the dyes are binding to endomembranes. We think it is not the plasma membrane because if it were changes in the plasma membrane we should see those over all regions of the cell. ANS, however, could be binding to microtubules. That is well known. Again, Steve Wolniak has used filters that enhance for membrane fluorescence.

When it comes down to actually explaining the cause of the observed changes in fluorescence, we simply don't know enough to ascribe a particular function. It could be a change in membrane potential. It could be a change in translocation of ions on the surface of the membranes. We are trying to be very circumspect about exactly what the changes are. What I do want to emphasize though is that we are seeing changes that are occurring in the spindle that are correlated with the events of mitosis and that it does not seem to be just a distributional change of membranes; rather there does seem to be some change of the ions within the milieu of the mitotic apparatus and to the best of my knowledge this is the first time that people have made such a correlation between ionic events and the specific stages and structure of the mitotic apparatus.

I know that my time is running late. I want to make a couple of comments about the final aspect of my talk, namely, if by modulating the calcium concentration, can we affect the events of mitosis. For these studies I have used dividing cells of stamen hairs of Tradescantia. What I have done is to modulate the calcium concentration using calcium EGTA buffers, and agents such as lanthanum, and D600 which block calcium channels. I also, tried A23187. The first three were used to lower calcium in the cell, while the ionophore was used to raise intracellular calcium. I have measured the time of nuclear envelope breakdown to anaphase, the time from the onset of anaphase to cell plate, and the rate of chromosome motion at anaphase. The results were surprising to me.

In controls, the time of nuclear envelope breakdown to the onset of anaphase is around 32 minutes. From anaphase to cell plate is 19 minutes, and the rate of chromosome motion is around 1.4 microns per minute. In cells that are soaked for some time in EGTA, and low calcium (10 millimolar EGTA, and 10^{-8} or less calcium) the time for metaphase is increased to 55 minutes on the average. The time for cell plate formation is still 19 minutes, and the rate of chromosome motion is still 1.4 microns a minute, and that is what really surprised me. I thought that I might be able to markedly affect the rates of chromosome motion. I am perplexed because I thought that if anaphase involves a breakdown of microtubules and that this process needs calcium that by blocking, or inhibiting the calcium here I might be able to slow this process down. That doesn't seem to be the case at all.

The A23187 experiments are confounded by the fact that I had two different batches of A23187 from Cal Biochem and I did not have, at that time, an independent assay for its quality. A23187 however, had no effect whatsoever. In other words they went like control all the way through.

I see that I have spent my time, and I thank you for your patience. I end up by simply saying that mitosis is an important process. It may be calcium regulated, but there are lots of holes, and lots of work yet to do on this, but I think that we are starting to piece things out, perhaps through the use of some of the probes that Roger Tsien will be telling us about we may be able to more directly look at free calcium changes.

That, to me, seems to be one of the key experiments. Can we, with aequorin or one of the quin dyes actually see changes in free calcium and if so, when and where they occur.

That I think is the most important question.

Thanks.

DR. ROUX: We have a couple minutes for questions before we have a break.

Bob?

DR. BANDURSKI: Are these fibers big enough to measure their strength --

DR. HEPLER: The spindle fibers?

DR. BANDURSKI: Yes.

DR. HEPLER: Bob Bandurski asked whether we can actually measure the strength of the spindle fibers. Nicklas at Duke and Salmon at Chapel Hill have done a variety of micro manipulation experiments where they measure the force of these fibers. I don't know what the figures are, but there is a length-tension relationship. The longer the fiber the greater the tension. That I do know. I don't know what the absolute values are.

DR. BANDURSKI: Any other chemical parameter like CD of the fibers or even spin labeling, anything to tell you what is happening to the protein, the chemical protein?

DR. HEPLER: Yes, Bob, again, asks whether there is any change in circular dichroism; are there changes that we could see in these proteins? I don't think those experiments have been done. They may be possible.

DR. KRETSINGER: I was intrigued by the cartoon that you had of the membrane distribution. I don't know if it is difficult to go back 12 slides and show it or sketch it on the board, but there are two implications there. One is that all the vesicles that have been referred to in the past, like you showed in a picture of the Arbacia which is just absolutely chock full of membranes seen in cross section, are in a sense artefactual. That is that they have closed after the preparation, but they are part of a continuous membrane sheath. That is what you meant to say?

DR. HEPLER: That is what I meant to say.

DR. KRETSINGER: Even that highly packed Arbacia.

DR. HEPLER: In Arbacia fixation is not as good as it is in plants. The membrane system in Arbacia is more swollen, and I tend to think that it has vesiculated in the fixing process.

DR. KRETSINGER: Okay, now, I would like you to elaborate just a little bit on the implied topology of that cartoon. As I understood the drawing there are two sorts of space, the space that is continuous with the cytosol and the space that is contained within the vesicle. As you drew it, the midline of the spindle, for instance, is part of the space that is continuous with the cytosol and this double membrane layered vesicular cap, the two of them are a space that is topologically distinct from the cytosol. Is that what you meant to apply?

DR. HEPLER: Yes.

DR. KRETSINGER: How good is the evidence for that?

DR. HEPLER: Can I elaborate on this?

DR. KRETSINGER: Please.

DR. HEPLER: This is a very important point that I must have gone over much too fast. Can we use the freeze fraction terminology, E space and P space?

DR. KRETSINGER: Sure.

DR. HEPLER: I would say that the space which is inside the membrane cisternal space is an E space. It is an extracytoplasmic or extra spindle space. That which is in the spindle is a cytoplasmic or P space, but there are so many membranes it is as if that particular cytoplasmic space is isolated from other cytoplasmic spaces. I would say that is a special domain, perhaps using the terminology of Rose and Loewenstein.

DR. KRETSINGER: Why is it isolated, that is the diffusion path is so long, it is structurally distinct?

DR. HEPLER: You do see a lot of membrane around it. Yes, there are pores in it, but there are pores in the nuclear envelope, too. I would say to the extent that a nucleus can be separated from the cytoplasm by its nuclear envelope, so, too, can the spindle be separated from the neighboring cytoplasm by this membrane sheath.

DR. KRETSINGER: But, again, from the drawing that you presented one would infer from that that the midline of the spindles was exposed to the same ionic environment as that of the cytosol.

DR. HEPLER: What I meant to say was that the spindle is continuous with the cytoplasm, but that you do have essentially a membrane sheath that separates the two. The membrane has fenestrations and discontinuities so that while there is a path, between the spindle and cytoplasm, it is probably tortuous.

DR. KRETSINGER: But still if you are saying in a topological sense they are part of the same space --

DR. HEPLER: Oh, yes, for sure.

DR. KRETSINGER: -- the same volume. And all your reconstructions or those that you know of are consistent with that.

DR. HEPLER: Absolutely.

The spindle is in the P space, not the E space.

DR. ROUX: Why don't we continue the discussion over coffee.

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HEPLER'S FIGURES

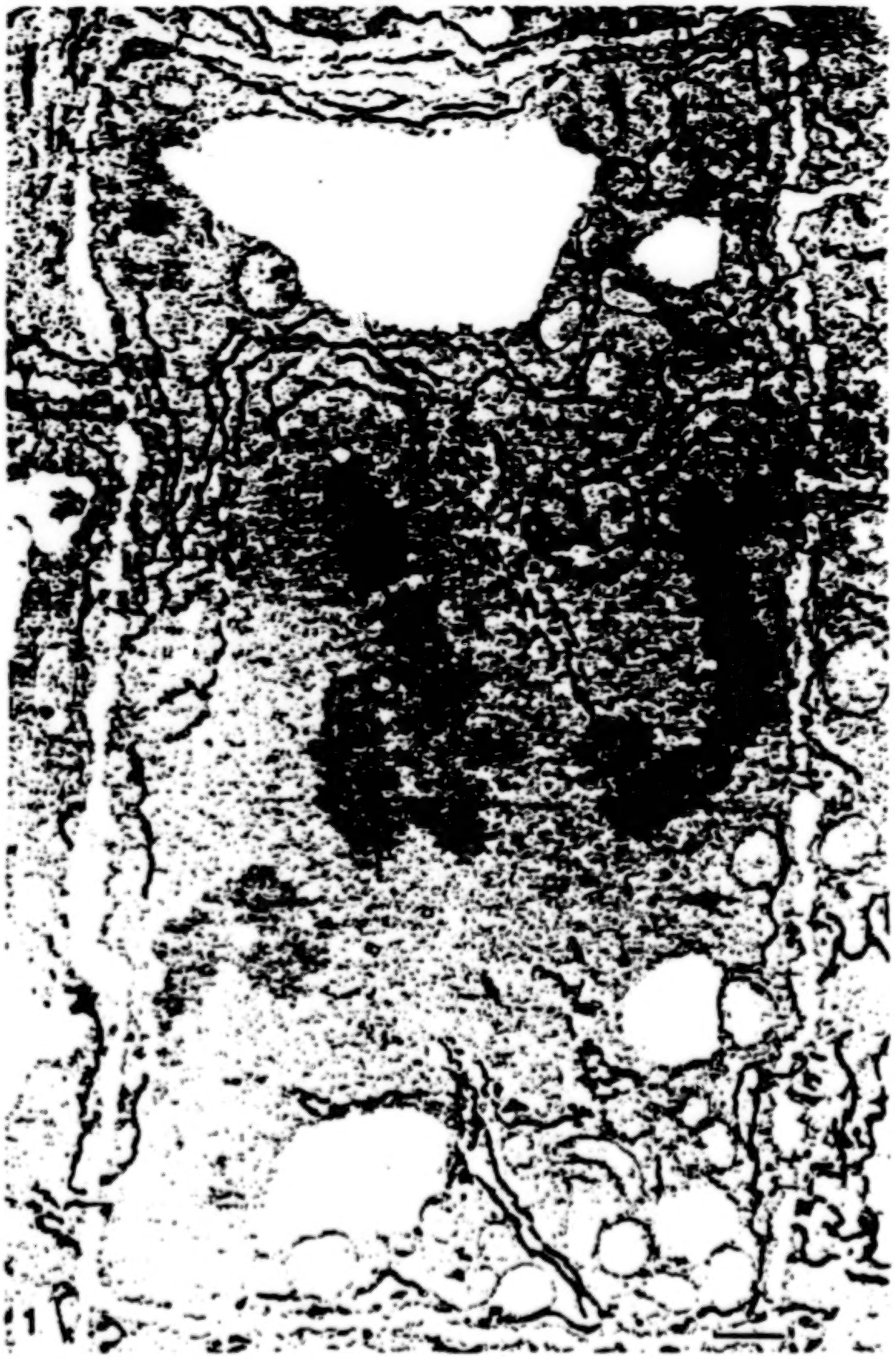

Fig. 1. Metaphase in barley leaf epidermal cell. ER has been selectively contrasted by the OsFeCN fixative. Prominent membrane elements occur in the upper spindle pole and extend into the mitotic apparatus making contact with the chromosome at the kinetochore (•). × 10.000. Bar = 1 μm

NOTE: Figures 1 - 4 are taken from Reference 3

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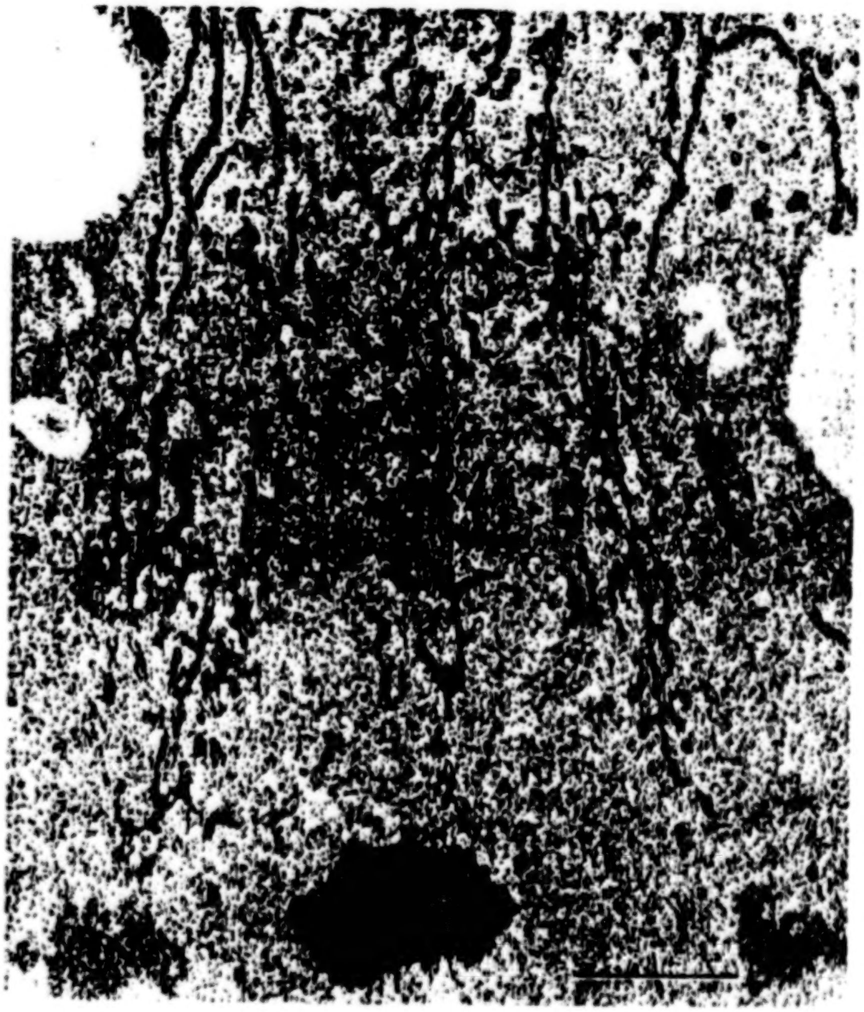

FIGURE 2. Barley leaf metaphase showing OsFeCN stained membranes associated with three kinetochores (*). This figure emphasizes the extensive interpenetration of ER into the mitotic apparatus. x25,000. Bar = 1 μm.

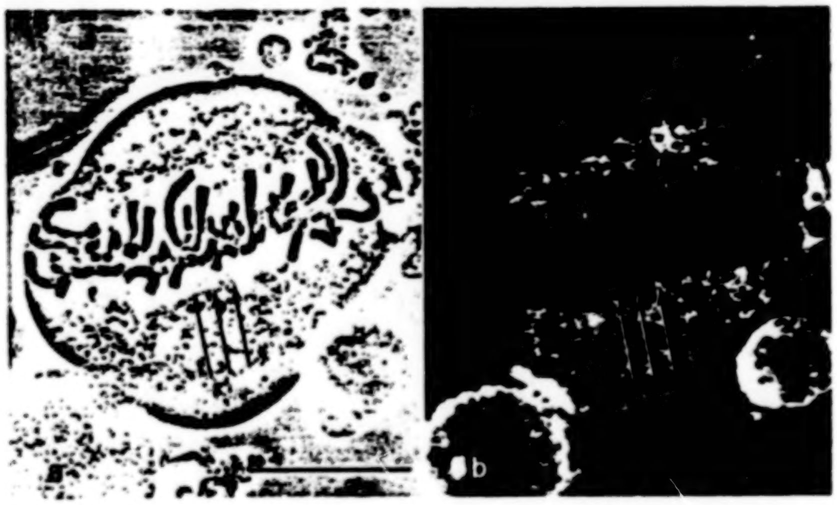

Fig. 3 Paired phase contrast (**a**) and fluorescence (**b**) micrographs of a *Haemanthus* endosperm cell in metaphase. The cell has been treated with 20 μM chlorotetracycline. Cones of diffuse chlorotetracycline fluorescence (*arrows* in **b**) coincide with the location of kinetochore fibers (*arrows* in **a**). Plastids and mitochondria are restricted to the spindle poles and contribute to the punctate fluorescence (From Wolniak et al. 1980) ×530. Bar = 50 μm

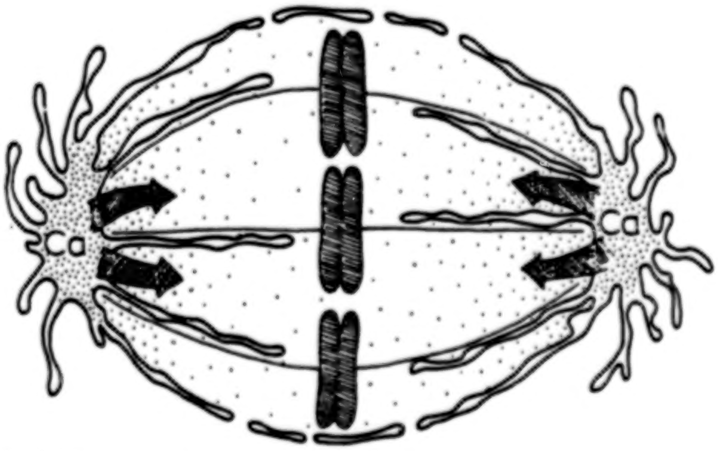

Fig. 4. Diagrammatic representation of a cell in late metaphase. An extensive, Ca^{2+}-containing membrane system occupies the spindle poles and extends into the spindle interior along kinetochore fibers. As the cell progresses from metaphase to anaphase we suggest that there is a release of Ca^{2+}, from the membrane compartment. The resultant increase in free $[Ca^{2+}]$ in the mitotic apparatus may activate one or more motile events associated with chromosome motion

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Figs. 5-8 . Fluorescence intensity changes during the metaphase/anaphase transition in living endosperm cells of Haemanthus. The onset of anaphase (time = 0) was judged visually by phase contrast microscopy. Values for fluorescence intensity were normalized to a percentage of the mean value obtained during metaphase (metaphase mean = 100%) [from Wolniak et al. (14).

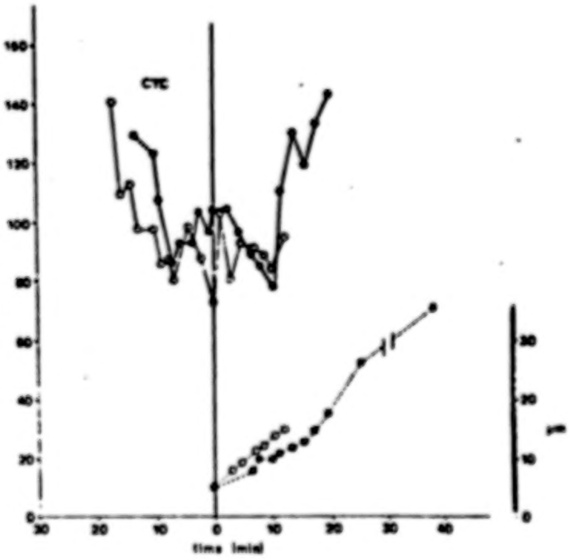

Fig. 5. Spindle fluorescence (solid line) from two endosperm cells (closed and open circles) pretreated 20 min with 25 µM CTC. Chromosome movement (dashed line) represents total separation of the centromeres.

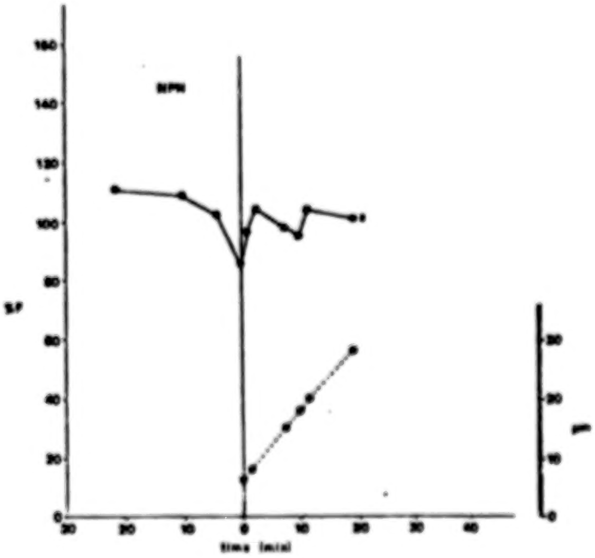

Fig. 6. Spindle fluorescence from an endosperm cell pretreated 20 min with 25 µM NPN. Chromosome movement (dashed line) represents total separation of the centromeres.

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HEPLER FIGURES (cont.)

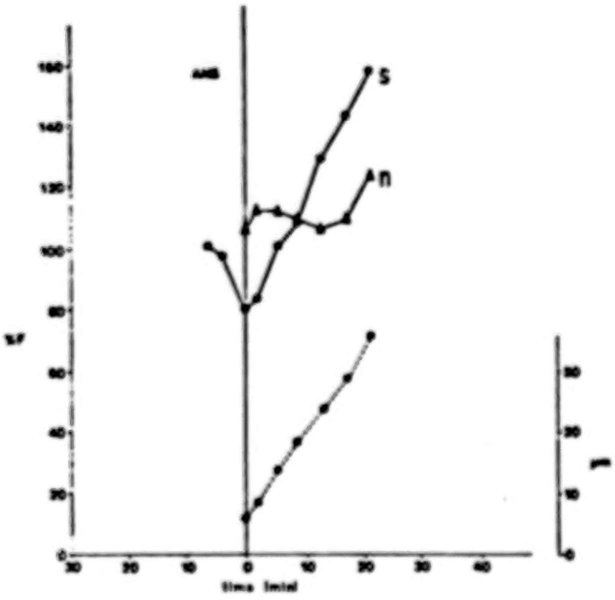

Fig. 7. Spindle fluorescence (s), and nonspindle fluorescence (n) from an endosperm cell pretreated 20 min with 15 μM ANS. Chromosome movement (dashed line) represents total separation of the centromeres.

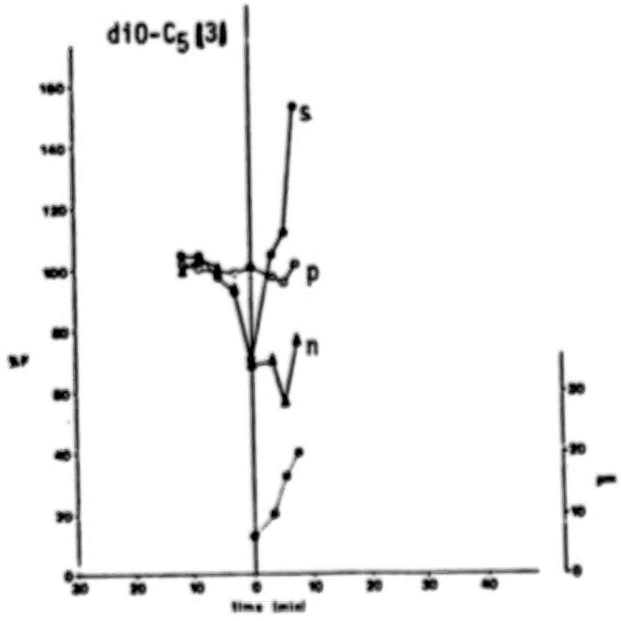

Fig 8. Spindle (s), nonspindle (n), and pole (p) fluorescence from an endosperm cell treated with 0.2 μM diOC$_5$(3). Chromosome movement (dashed line) represents total separation of the centromeres.

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DR. ROUX: If we could start the second half of the program, I would really appreciate it.

Okay, the first speaker after the break is Dr. Charles Caldwell who is from the USDA in Beltsville, and the topic of his talk will be the calcium modulation of plant plasma membrane bound calcium ATPase activities.

Chuck?

DR. CALDWELL: Thank you. I, too, do not consider myself a calcium specialist, and I should preface my talk by emphasizing that in spite of its title I would be reluctant to claim that I am working purely with plasma membranes from plants. Stan has alluded to this earlier. Much of the work is done with crude microsomes, and we try further to purify it. The problem is, unlike the animal membrane systems, and I am very envious of those of you who work with animals, that we don't really have unambiguous markers for plasma membrane. Various people have claimed hormone binding sites, certain other properties are only found in plant plasma membranes, but there are still problems in verifying purity.

Most of my work is with barley ATPase. I have found myself in the perhaps not enviable position of being in between plant and animal in terms of kinetics, since my early background was in animal physiology, animal biochemistry. So when I started to look at ATPase activities purely as an alternative to some biophysical measurements using EPR, I went to the animal literature first before going to the plant literature and started doing experiments without really having, unfortunately, looked at what people had been doing in plants, particularly work of Tom Hodges and R. T. Leonard. As a result, when I did my first experiments the kinetics were bizarre for a plant. I will be spending some time speaking about the properties of barley enzyme so that I can compare the properties of the barley enzyme to other plants. In many cases, though it is not quantitative or qualitative, the barley ATPase is quite similar to some of the animal enzymes.

As I mentioned in the beginning, the first problem one has in looking at plant-associated membrane function is to isolate the membrane.

A picture of a typical plant cell gives you an indication of one of the problems one comes across. The earlier speakers have mentioned the vacuole being a large volume. I prefer to think of it as the cytoplasm being a very small volume. There is often a very small bit of cytoplasm between the vacuole and a very thick cell wall. So when you isolate membranes you have a number of alternatives as to what you start with, and most people or many people use intact tissues which you homogenize and then do differential centrifugations and sucrose gradients. Others have bypassed part of the problem by using enzymes to digest away the cell wall, making a protoplast which in turn can be used either as an intact system for studying transport and as a source for membranes for subsequent fractionations.

Since I am interested in plasma-membrane biophysics, as well as, calcium transport, I was reluctant to go with the protoplast system, since the enzymes used to digest away the cell wall are crude and contain proteases, lipases, etc. In fact, people who have looked at protoplasts with the ability to bind radioactive labeled hormones, in some cases when the protoplast is first made there is no apparent high specificity binding, but after a period of time the protoplasts, which stay viable, gradually acquire the ability to bind hormones which suggests perhaps that protein is being synthesized and reinserted. This by inference means they digested away at the initial stages but that is still open.

I found references which indicate that calcium can get into the vacuole. In a duck-weed cell, and this may be a somewhat unique situation, one finds vacuolar membrane, protoplast membrane and calcium oxalate crystals. Ledbetter and Porter claim that these crystals develop in certain discrete regions. You see small membrane encapsulated structures. As the calcium oxalate develops it gets subsequently put into these larger structures, but still have membrane holding them together. Apparently old leaves have a tendency to accumulate calcium oxalate as well. I doubt if this calcium would be available for the type of processes that Stan has been talking about. However, it was interesting that Ledbetter and Porter predicted presence of a calcium pump somewhere in this region. It would be nice to think that this membrane would encapsulate these crystals as they develop. It has recently been shown that there is ATPase activity associated with protoplast membranes that could facilitate calcium movement.

So, I think the best way for me is just let me go through quickly some of the kinetic properties of barley enzyme and compare it to that of other plants. The following are two alternative methods for calcium transport in plasma membranes or in other membrane structures. The first one is essentially redrawing the figure by Erasmo Marre' where he is suggesting that there is a proton pump (Figure 1). There is a lot of evidence for ATP-dependent proton movements next to the membrane vesicles. Proton pump is energized by the hydrolysis of high-energy phosphate. I did not say ATP here because there is some question as to what can be utilized. Some of these enzymes seem to like ADP as well. The calcium efflux is then through a counter transport, antiport system about the cell or maybe a potassium uniport, but seems to counteract or is an obligatory process related to the proton movement. There is evidence, this is mostly from in vivo type studies in barley and other plants that there is a hydrogen-sodium exchange, an amino acid sugar cotransport and anion transport systems.

In looking through some literature in preparation for my talk today, I came across a figure illustrating Streptococcus transport back in 1972. It was interesting because they were comparing it to what they know now about the transport in Streptococcus. Earlier they had a single proton pump. They now have five different ATPases of which the fifth one they are still trying to find out what it does.

The second possibility for calcium transport is based on the work of Dieter Marme', and hopefully I will remember to say the first names of about three scientists, Erasmo Marre', Dieter Marme' and Jim Morre'; so you will keep track of who is who. Dieter Marme' has proposed that there is an ATP-dependent directly coupled calcium pump in plasma membranes themselves (1). He has used crude microsomes during much of his experimentation, but there is a very definite ATP-dependent calcium movement in the microsomal preparations from corn and other plant species. He has isolated the ATPase protein, after solubilization, by affinity chromatography and found that it has a molecular weight about 100,000 Daltons. In some respects it seems very similar to calcium pump of some animal membranes.

With barley it is possible to solubilize the membrane using detergents in the presence of glycerol and running through ATP affinity columns. This gives a phosphorylatable protein of about 100,000 Daltons. My staining never did work very well because in my case I was putting lipids back into the system to get maximal activities. Many other people have not put lipids into the system. It turns out that with the silver staining procedure

I was using the lipids stain the whole channel black, but in terms of P32 incorporation there is a single hot band about 100,000 Daltons. Much of the earlier work with plant ATPases involves corn and oat, though many other plants have been studied in the past five or 10 years. Though it is not unique to barley, one thing that struck me as being considerably different from other plant ATPases is the fact that calcium markedly stimulates enzyme activity. This was done by having constant concentration of ATP and simply adding divalent cations. It seems to like calcium a lot. Also, at high superoptimal concentrations calcium seems to be inhibitory. The ATPase likes magnesium somewhat, but other ions, including zinc, nickel also seem to activate the enzyme. Since there have been reports of divalent cation independent ATPases, I decided to check the effect of chelators on the enzyme activity, and there is a decline with both EDTA and EGTA. It would seem as though there is calcium around. I would doubt if I could ever get rid of it from glassware, in fact, ATP itself from Sigma had a lot of divalent cations associated with it. It is possible to clean it up a bit better now. The further decline to zero appears to simply be an artefact chelator effect on the phosphate assay. So, at least in barley it looks as though calcium is the preferred ion. This is different from the calcium ATPase activity of animals which still uses magnesium as the substrate activator.

Some people question why barley should be so different from other plants. Now, it made some sense that barley has a calcium activated enzyme because barley tends to like calcium. It grows well in calcium-containing soils where oat, which has a high magnesium activity, tends to dislike calcium-containing soils. Kuiper and Kylin and other workers (2) in Europe have shown very nicely that certain plant varieties that prefer to grow in different types of soils have different ATPase activities with respect to divalent cation dependencies.

If there are in fact, two ATPases in the plant plasma membranes I wondered why in most of the kinetics for plant ATPases, one gets simple Lineweaver-Burke plots, that is, straight lines. Although there are circumstances kinetically where one would have two enzymes giving one straight line, it bothered me quite a bit. So, when I did it the first time I kept getting kinetics like this. These are Lineweaver-Burke plots for the ATPase, using calcium ATP^{2-} as the presumed substrate, and magnesium ATP^{2-} as the presumed substrate (Figure 2). I spent six months trying to make these into straight lines, and I had a lot of difficulty doing it. The top curves were obtained using the method of millimolar ratio for preparing substrate. Assuming the substrates are the divalent cation-ATP complex in the 2 minus form then one has to add magnesium or calcium and calculate the substrate concentration. In animal systems it has been shown at least theoretically, that the one-to-one molar ratio method is probably not the best to use, because it results in high concentrations of free ATP in various ionic forms. So, when one does saturation kinetics it would be nice to have only one variable, which is the presumed substrate. When you use the one-to-one molar ratio at pH 8 you don't get it. Therefore, at pH 6.5 where most people do the plant or ATPase assays it is certainly a problem. I have seen occasional reports where the authors claim that when one puts in 1 millimole of ATP and 1 millimole magnesium at pH 6.5, 95 to 100% of the ATP is ATP 2 minus. When I do the calculation using a variety of methods, it seems to be about 40%. So this can make a big difference in kinetic constants. A molar excess method, where I added an excess of the divalent cation was preferable. There are obviously difficulties, particularly because the divalent cation concentrations, free divalent cation concentrations calculated at least are at levels which would be somewhat inhibitory to the ATPase activity. In other data I have found that free ATP is very inhibitory for ATPase activity in plants, at least in the barley membrane. So, it would seem preferable to maintain constant levels of all the potential inhibitors and only vary the substrate concentration which occurs when you use the millimolar excess method.

Kinetics, like I mentioned earlier, are not common to all plant ATPases. So, I was curious why barley is so different, other than the fact that it has high calcium-dependent ATPase activity. Other plants which also have high calcium-dependent ATPase activities and tend to grow in high calcium soils including horse bean and to some extent wheat, have ATPase kinetics which seem to be straight lines.

Now, having gone to the animal literature first it seemed logical to study my ATPase at a temperature I grew the plant, which was 16 degrees centigrade. I used isolated membranes from five-day-old barley seedlings. Most people or many people working with plant ATPases study them at 37 degrees centigrade. I guess it is because that is the temperature at which the people working with animals study them as well. When one looks at the temperature dependence of the ATPase kinetics in barley, one can see a certain degree of linearization of the plot as a function of temperature. The number in parentheses is the Hill coefficient. This is not meant to indicate that I think this is a negatively cooperative enzyme, but simply is used an index of linearity. Even though I can see some degree of non-linearity down this region, this perhaps would not be as evident under the conditions where many other people study their enzymes. So, at least in terms of barley activity, I cannot say that my biphasic kinetics is unique to barley.

Recently in plants there have been some indications with Limonium root membranes that there is a high and low affinity component and more recently Don Briskin (3) who is doing a postdoc in Montreal has found that the Km for phospho-enzyme formation in corn is about 20 micromolar. When one calculates the kinetic constants for these type of curves using the non-linear squares method, it turns out the high affinity for barley in the presence of calcium is about 15 micromoles. So, at least it is in the right range for phospho-enzyme formation and apparently, as he says the phospho-enzyme formation is the rate limiting step, one would expect that the Km for the total process might be in that range. He, also, says that when one goes to higher substrate concentration, one sees a second phase of phospho-enzyme formation.

Now, whether or not this represents two enzymes or a single enzyme which has negative cooperativity I really cannot say. In barley, if one grinds the roots, solubilizes the membrane with salts and runs them on electrophoresis, staining for phosphate release from ATP, one gets a number of bands. As I recall, Hall had six or eight ATPases, which suggests that there are perhaps multiple phosphatases. Whether or not they are all specific for ATP remains to be seen.

I have examined the pH dependence of the enzyme. Once again, since I have two components I have done all the analyses based on the assumption I have two enzymes. Until I can prove otherwise I will continue with that. In barley, Nagahawshi (4) has found that at high substrate concentrations pH optimum for the ATPase is pH 6, the same as my low affinity component. The high-affinity component seems to have a pH optimum around 5. Now, this is different from that which is often reported for plant ATPases at pH 6.5. If I take barley and run the ATPase assays at high substrate levels, I do get a single optimum around 6. It is equivalent to the results of Nagahawshi. However, since the substrate is presumably magnesium or calcium ATP-2 minus, as one goes from pH 6 to pH 5, the formation of the other ATP complexes seems to reduce ATPase activity. So, if one really wants the enzyme pH optimum, one should correct for substrate ionization. Now, one could argue what is the relevant pH optimum, since in the plant both the substrate and the enzyme catalytic sites are exposed to this pH. Nitrophenylphosphatase activity does increase with decreasing pH, but it doesn't seem to have an optima at the same places as the high affinity component site. I think the non-specific phosphatase might still be a problem in these systems, but I don't think it causes the pH 5 optimum.

Analysis of the ATPase Km's, using the method of Dixon, indicates that at the most physiological pH's, enzyme-substrate binding is by charged neutralization. This may be of some relevance in looking at the effect of divalent cations on enzyme activities or perhaps anions on enzyme activities, if these can bind to the catalytic sites.

Figure 3 shows the temperature dependences of the enzyme Km's. I am using KH to indicate the high affinity component and KL the low affinity component. At least from the 15 to 35 degrees, the Km's of the calcium- and the magnesium-dependent activities seem to be fairly constant. There is a major decrease in enzyme substrate affinity about 35 degrees here and about 14 degrees here. At least over the range of 15 to 35 degrees for the high-affinity component, the Km is relatively constant. To study enzyme temperature dependences, one always does Arrhenius plots. Figure 4 shows the maximal velocity once again. These are not single substrate concentrations. I like to emphasize that because it took me 30 hours to do each experiment, and I like people to realize how much trouble it was. To me at least these are not two straight lines but curves. There is some argument as to why one would get a discrete break. I have drawn the lines here just to show that temperature that would come out is around 20 degrees centigrade, which is the temperature at which a lot of plant and animal ATPases have discontinuities. I have used a mathematical relationship proposed by Silvius and McElhaney (5) for the analysis of thermo-dynamic contents of Arrhenius plots. Also, using the logic of Reed et al. (6) which is that one should not try to fit points which are resulting from Km changes. Since as you may recall from the previous slide, there was a Km change around this temperature, I have only used these points to fit the curve.

I came with a lot of numbers, heat capacities of activation, activation enthalpies. I am not showing them to you because I don't know what they mean. The heat capacity of activation for all four figures was about minus 750, but that doesn't really, to me at least, mean anything. There is no theory which would explain why it should change or why it should stay constant as a function of temperature.

Recently there has been a lot of work using some variety of methods of membrane isolation, in particular the dextran cushion method as suggested by Heven Sze (7). What she found is that there are vanadium sensitive and vanadium insensitive ATPase activities in plant membranes. Therefore, I wondered if I could distinguish between the two types of ATPase catalytic sites in barley membranes by the presence of vanadium. The open circles in Figure 5 are the magnesium dependent ATPase activity. In general, the plant ATPases are somewhat less sensitive to vanadium than the animal enzymes. If one adds vanadium at 100 micromoles, a straight line is obtained. There are some indications of small changes, as one might expect with the presence of a second enzyme, but if you look at percent inhibition of ATPase as a function of substrate concentration, once again at 100 micromoles of vanadium, it looks as though maximum inhibition occurs at low substrate concentrations. So, I really cannot say this proves there are two enzymes because Bowman and Slayman (8) with Neurospora have found that the Neurospora ATPase seems to be positively cooperative. That is, instead of bending down here, they bend out and when one adds vanadium they become straight lines. They have been able to purify the enzyme to homogeneity. I don't recall if they did the vanadium experiment with purified enzyme or with the Neurospora membrane isolates. Nonetheless, it looks as though they have a single enzyme, and they can straighten their kinetic curves by adding vanadium.

The lower closed circles are the calcium effect. The calcium ATPase is not as sensitive as the magnesium. Once again, I cannot attribute that to the presence of a calcium enzyme and a magnesium enzyme simply because calcium in Neurospora and apparently in erythrocytes as well tends to protect the enzyme from inhibition by vanadium. What is interesting is that maximal inhibition is about 50 percent of enzyme activity. Going to the literature there seemed to be some dispute. Perhaps somebody here can tell me later what people finally decided about whether or not the directly coupled calcium ATPase or calcium pumps are really sensitive to vanadium or not. I would like to say I could study the barley calcium pumps as if they are the same as the animal by putting in vanadium which would mask this other activity. That is still open.

All the work I have done in terms of the effect of ions on enzyme activity has been based on the premise that one should not ignore the lipids. The lipids are charged negatively and the addition of excess divalent cations, as shown on the first slides on the activation by calcium, can inhibit enzyme activity. It has been shown in animal membrane systems that excessive amounts of calcium inhibit enzymes perhaps by direct binding to lipids. It is an indirect phenomenon related to lipid interaction with membrane bound enzyme.

I have used electron parametric resonance or EPR to study two phenomena. This is a test probe I am using. It is called CAT12. It is cationic and has a chain of 12 carbons. This is the reporter molecule, the nitroxide radical. Based on the assumption that it is an amphile and that it is going to situate in the membrane probably with the acyl chain end, the radical should be up near the region of the head groups. Other membrane probes used have reporter molecules down on the acyl chains of stearates, for example, about the 12 position, etc. That would measure things down into the bilayer.

Based on breaks in Arrhenius plots, many people predicted the presence of lipid phase transitions in plant membranes. I realize that to any physical chemists here there is no such thing in biology, but the solid to liquid melt based on chain lipids controls enzyme activity at these breaks. That is what these breaks suggest. This was the consensus in plants until recently when it was found with calorimetry and using other probes that, at least in barley, the phase transition is complete when one reaches minus 6 degrees centigrade. So, the bulk phase transition is over before you get to 16°C.

So, with this probe it is possible to measure two phenomena. I like to be able to measure two different things using a single signal because it does take some time to run these experiments. The first thing one generally measures is mobility which is a reflection of the distance between that peak and that trough in gauss. This is the first derivative spectra (Figure 6). The value of 2T parallel is dependent upon the mobility of the probe in its local environment. If this probe happens to be localized in a certain area of the membrane, then I might be getting some artificial measurements of mobility.

Since CAT12 is an amphiphile, it partitions between the aqueous space and the membrane. One can measure the membrane signal, which is this broad peak. The trough and a portion of the center signal is also from membrane bound probe. These sharper signals and, also, a portion of this inner signal is from aqueous probe. So what one simply does is measure the partition coefficient by taking the ratio of membrane bound signal to free probe signal. Since one can really not quantitate the free membrane surface potential, one measures relative changes in membrane surface change or delta psi by the addition or absence of calcium. For example, this one A, is in the absence of calcium; B, is in the presence of calcium. So you see a shift of increasing 2T parallel by a rigidification of the probe signal and, also, changes in the partition of the signal because it is being actually driven out of the membrane by the calcium association with the lipid head groups.

This is the 2T parallel as a function of temperature. There are apparently changes in the rate of 2T parallel value change as a function of temperature at about 10 degrees and about 14 degrees in the presence of calcium. This would indicate that the membrane is more rigid at higher temperatures in the presence of calcium, which is very consistent with data from animal membranes.

People in the past had trouble with the unit 2T parallel. It changes by a factor of two. What does that mean? I have used a different type of unit which is mostly for my own benefit. It is called equivalent temperature change (ETC). By the addition of calcium these are the ETC values, the closed circles. The membrane gets more rigid. That rigidity is equivalent to a change in temperature of minus 2 degrees centigrade. So, in other words, I have converted gauss to temperature change. All these experiments have been done isothermally at 16. So, in other words by the addition of 10 millimoles of calcium, according to CAT probe, the membrane is as rigid as it would be at 6 degrees centigrade versus 16. One can see differences using different probes; this is CAT 12, the one I showed you before, 5NS is the probe at the 5 position of stearate; 12NS is the 12 position of stearate. As one gets deeper into the membrane, these stearate molecules are assumed once again to be amphiphiles with the nitroxide at different depths into the membrane, the change in the signal induced by calcium is decreased. That would suggest there might be a gradient of probe mobility as calcium binds to the membrane surface. The deeper one gets into the membrane the less change one observes. The ATPase inhibition seems to follow the general modification of membrane mobility. This is at super optimal concentrations of calcium.

A problem with calcium is that it can do more than one thing. Calcium has the ability to both bind and screen lipid charges. We will assume that the open circles in Figure 7 are monovalent cations and the ones with the crosses in them are divalent cations. Screening ions do not actually bind to the charged lipids. They sit at some distance above the surface, but they effectively reduce surface charge. Now, how far up? Well this is dependent upon the water, the hydration of the lipid head groups, ionic strength, and the amount of the ion. Although monovalent cations can bind to lipid structures, most monovalent cations generally screen charge versus binding, where the ion is actually at close proximity to the negatively charged phosphates of the lipids and can displace water from the lipid structure. Bound ions tend to make the membrane more rigid, that is they immobilize the movement of the head groups. Screening ions tend to make the membrane more fluid, probably by changing the pK's of the lipid phosphates causing a release of divalent cations absorbed to the membrane.

One could predict, therefore, by the addition of ions which could be both screeners and binders, such as calcium, a number of different mechanisms by which the enzyme can be inhibited. The easy one is that calcium binds, makes the membrane more rigid, equivalent to a reduction in temperature, and simply reduce the V max or the velocity of the enzyme activity. Alternatively, the substrate has a negative charge. So, if one reduces surface charge and makes it more positive that would allow more substrate to be near the active site, activating the ATPase.

To test these hypotheses, I have tried a variety of ions which have different effects on membranes. Figure 8 is even worse than this one or better, depending on your attitude about it. This is the effect of calcium on both ATPase activity (triangles), on surface charge (open circles), and on the mobility or ETC (closed circles). As you can see enzyme inhibition by high calcium concentration really follows both of the two alternatives. You really cannot say that that inhibition results from a screening effect or a binding effect or alternatively the direct association of calcium with the enzyme itself. However, strontium is known from animal literature mostly with liposomes to principally screen lipids. It does not really bind very well to phospholipids. This is evident by the fact that one sees a very large change in surface potential but, also, one sees no change in mobility of the probe in the region of the head group and no inhibition by the enzyme activity. This was somewhat strange because I know in some systems strontium actually can activate ATPases by serving as an alternative to calcium. I can see no indication that strontium in the absence of calcium resulted in any enzyme activity at all.

Uranyl ions have a very high tendency to bind to lipids. It seems to follow quite well that the inhibition follows decrease in mobility. There is a screening effect at high concentrations. I also tested mercury, which I suspected would associate mostly with the catalytic side because there is considerable evidence that ATPases have an essential sulfhydryl group. The ATPase is inhibited at very low concentrations. It is inhibited starting at nanomolar concentrations, well before one sees any changes in membrane properties. So mercury is certainly an example of an ion which inhibits by direct interaction with the catalytic side.

I also tried cadmium (Figure 9) because of its relevance to stress physiology in plants. Cadmium seems to inhibit mostly by the binding phenomena and not the screening phenomena. What I was curious about, especially for some work I am doing now with aluminum, is what happens when one adds back in monovalent cations to a system which already contains cadmium. By adding sodium, you get a partial reactivation of the enzyme (Figure 9). The effect of sodium is to continue the reversal of surface charge, as one might expect by having a small ion and also, increased lipid mobility. In fact, it is even to a position where it is as though the membrane were at higher temperatures than at 16°C; so it is, in this case almost to 20 degrees centigrade. Ethanolamine also does very similar things. It reactivates the ATPase, although this could be a superimposition of another enzyme altogether. It, also, changes the degree of membrane mobility, making it more fluid or more mobile. It has a small effect on surface charge. I found out later there are some problems with ethanolamine because it tends to interfere at higher concentrations with the phosphate assay. Choline, on the other hand, affects surface charge, but has no effect on ATPase activity. It doesn't reverse the inhibitions or mobility. I was intrigued by this because the earlier work by Ratner and Jacoby (9) with a variety of plant ATPases, corn and barley and a few others, indicated that the activation of the enzyme by monovalent cations is non-specific, that is TRIS activates, ethanolamine activates. One of the contentions is that the potassium activated magnesium dependent ATPase in plant membranes is the transporter for the potassium. I wonder if this type of phenomena may be quite common, especially if there is a problem with one's ATP. It seems like every year they come up with something new in commercial ATP that we want to get rid of. First it was vanadium, and now, it seems to be aluminum. Since the aluminum inhibitions are, also, reversed by these ions, then perhaps some of the activation I am seeing with monovalent cations is a reflection of this inhibition by contaminating metals. If I want to get potassium activation of the ATPase, I just need to dope my membrane system with about 10 micromoles of aluminum, then I can get stimulation in the range of 2000 percent. So, that is remarkable but it is a reflection of the fact the enzyme activity has already been partially inhibited by the presence of aluminum. As I recall from an Analytical Biochemistry paper, aluminum can be as high as 1 mole percent of commercial preparations of ATPase.

The little I have done recently on calmodulin is that barley does seem to have a calmodulin activated ATPase that is found in the presence of magnesium and calcium (Figure 10). There is calmodulin stimulation apparently with the magnesium alone and with calcium alone. The upper curve is inhibited completely by chlorpromazine. I have no idea why the magnesium activity goes up unless, as I said earlier, there is calcium around I cannot get rid of. Calmodulin from barley or bovine brain are almost identical. The problem I had with this experiment is that it must be done within five minutes after isolation of membrane. I realize those of you who work with plant plasma membranes know it takes a period of time to isolate them. If I let the membrane fraction sit in the presence of low concentration of calcium, 50 micromoles, for more than 15 or 20 minutes, I cannot find any calmodulin activation at all.

I should emphasize this is a relatively small component of the total ATPase activity. In the presence of calcium I get ATPase activity in the range of 120 micromoles per milligram per hour, and here we are looking at 10. What I was wondering is what could be happening in the presence of calcium over that 15-minute period which would cause complete inhibition of this enzyme activity? In the literature, particularly the work of Jim Morre', there are reports of potent phospholipases present in most plant microsome plasma membrane preparations (10). They seem to be calcium activated. I don't know if they are calmodulin activated, too. When one solubilizes the membrane using Zwittergen and does not add back in lipids, there is no activation by calmodulin and there actually is no calcium and magnesium activity by the classical definition. When one adds back in phosphatidlycholine, one suddenly begins to see calmodulin activation. One, also, sees calmodulin activation in the presence of asolecthicin, which is crude soybean phosphatides containing phosphatidlycholine. So this, to me, at least, was quite analogous to the erythrocyte ATPase which has been purified by Gietzen and Niggli where you need to have phosphatidlycholine around to maintain calmodulin ATPase. I was somewhat surprised that serine did not give me higher values, but phosphophenylamine was the best in reactivating the calcium dependent activity.

I want to briefly mention an ATPase system in pea stems, as proposed by Morre' (Figure 11). I have added two other things to it. The Pacman is a phospholipase, chewing off the lipid head groups. It seems to be calcium activated. I am not saying this is a particular type class phospholipase and according to Jim Morre' it is activated by plant hormones. The hormone activated component seems to result in a complete loss of, not complete, a considerable loss of phosphatidylinositol. Figure 11 also shows fusicoccin (FC) binding protein that Marre' has worked with. It seems to be integral, that is, it requires detergent for solubilization. However, the ATPase component can be released apparently by the addition of salts, sodium chloride and sodium perchlorate or by the absence of divalent cations. So these are magnesium ions holding it together. In barley, if one isolates the membrane in high magnesium and treats with potassium chloride, about 90 percent of the ATPase activity is solubilized. This is consistent with the work of Marme' who finds that before he isolates ATPase by calmodulin chromatography, salt treatments cause a reduction of 95 percent of ATPase activity. Therefore, it suggests that the ATPase, the big ATPase in plants is probably, a peripheral protein. This enzyme has been purified by various methods. There is some disagreement of whether or not it is ATPase or ADPase, EC3615.

If you ask me questions about this later, I will talk to them. So, that is it.

DR. ROUX: We have time for about one question.

DR. KRIKORIAN: I am just curious. You were talking about the plasma membrane in this situation. Can you do equivalent experiments on the vacuolar membrane?

DR. CALDWELL: I wanted to talk about that, but I ran out of time. Heven Sze has been working with tonoplast membrane recently using various methods of purification and it seemed as though it has a proton pump in it. It seems like it might pump calcium, but this work has only been really developed in the last year and it is vanadium insensitive, so that opens some possibilities in my system and perhaps my vanadium assisted component is tonoplasts. It very easily could be.

DR. ROUX: Thank you, Chuck.

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Caldwell Figures

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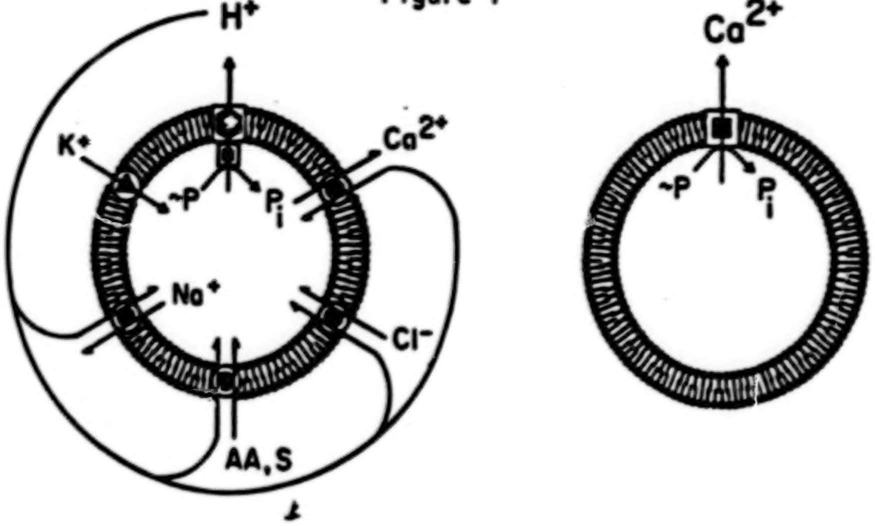
Figure 1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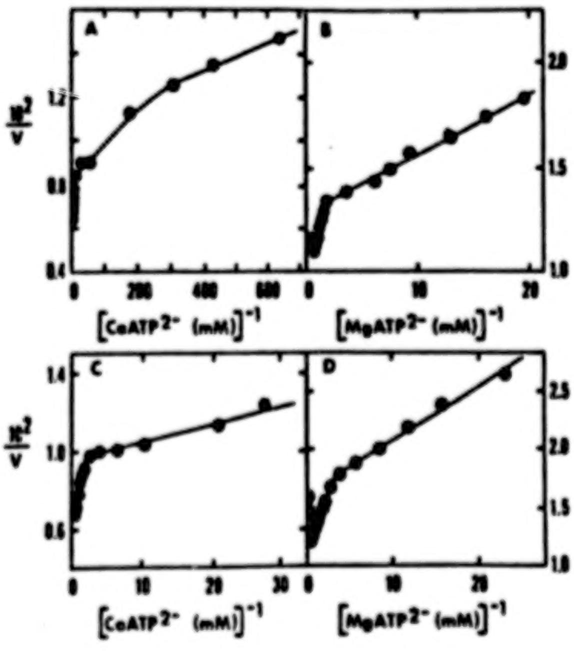

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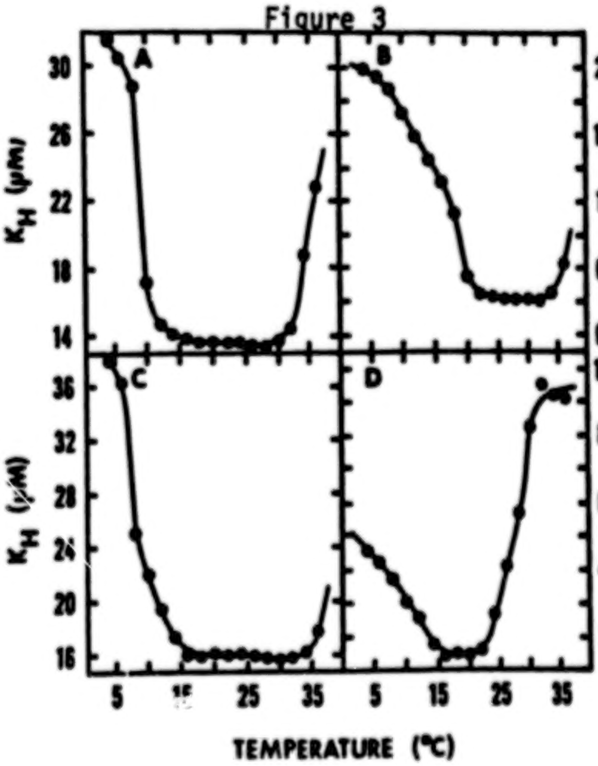

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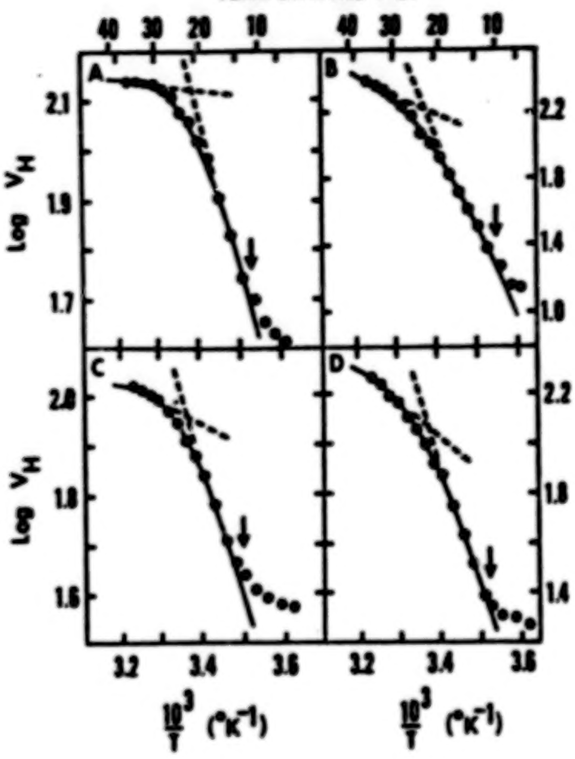

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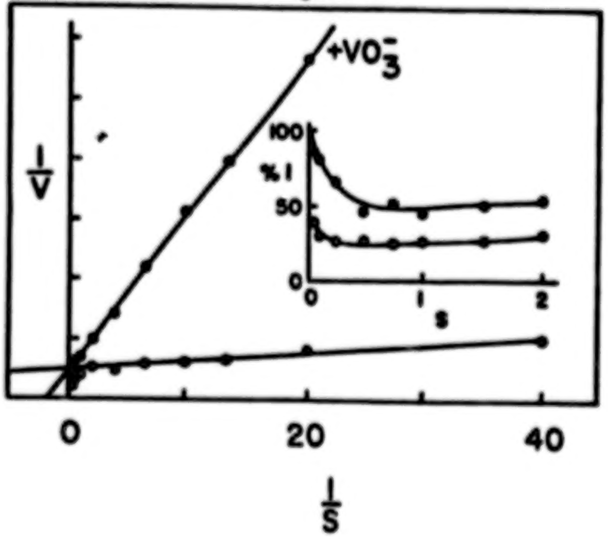

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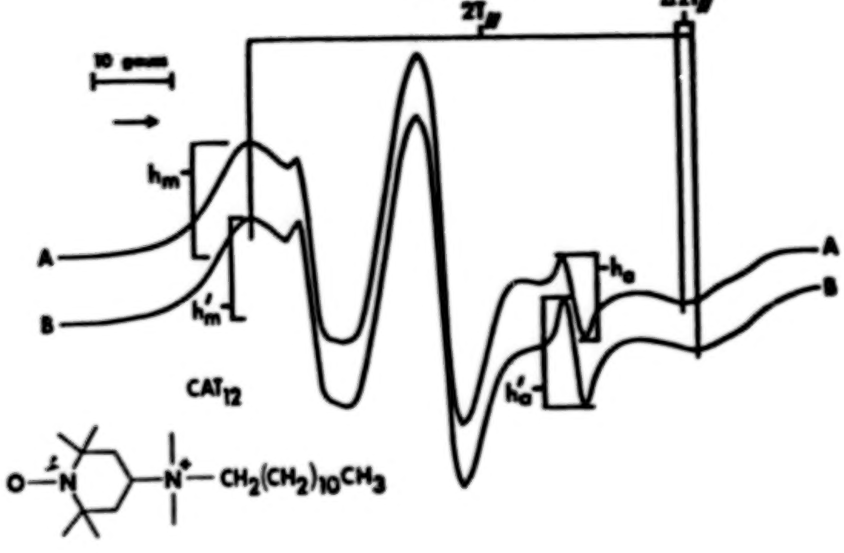
Figure 6



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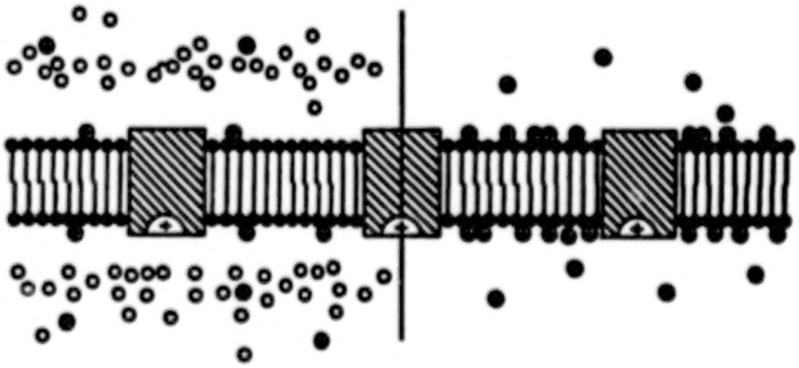

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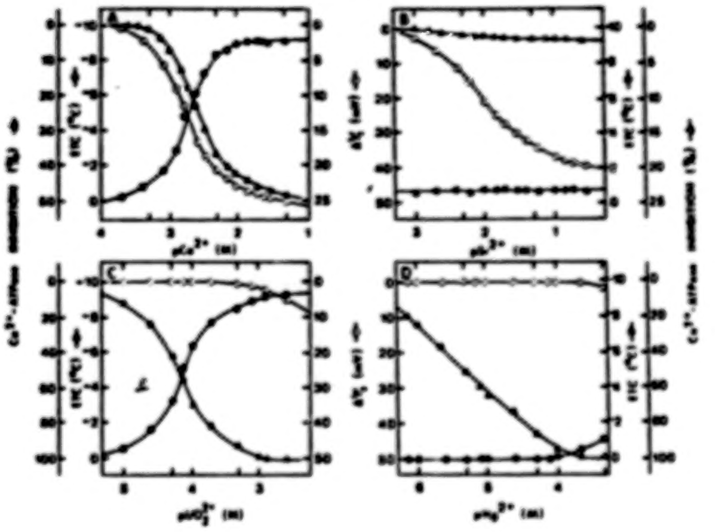

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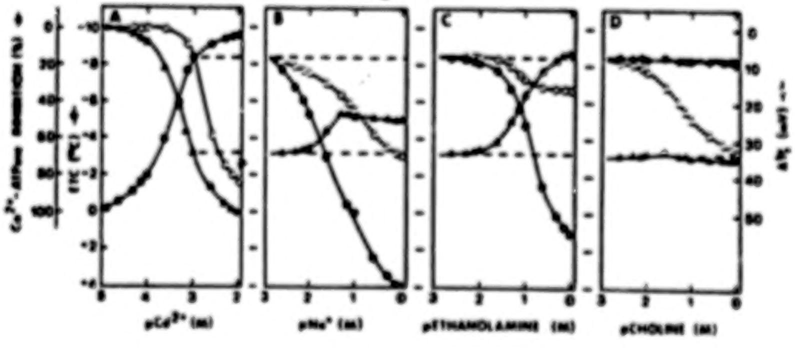

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Figure 10

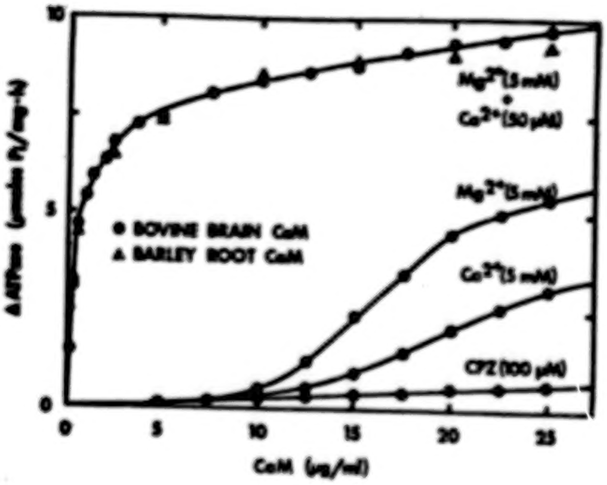
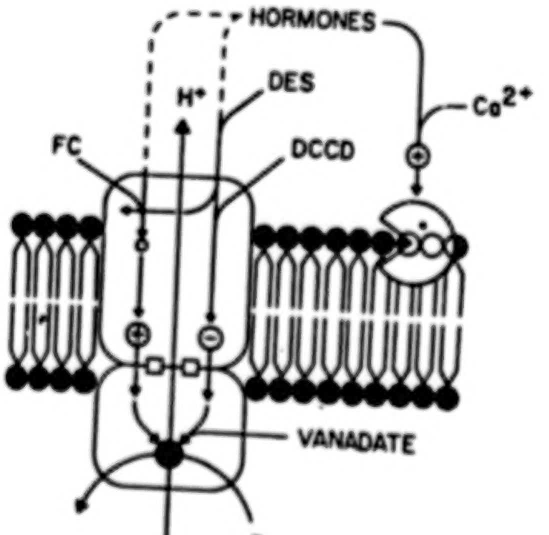


Figure 11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DR. ROUX: The last speaker before the lunch break will be Marty Watterson from the Howard Hughes Medical Institute at Vanderbilt, and he will be talking to us about intracellular calcium receptors, calmodulin and related proteins.

DR. WATTERSON: Stanley asked me to give a talk to a group of space biologists. I was not quite sure what I should cover. So, he gave me some guidelines. Also my children pointed out to me that there was a recent article in Science 1982 about the NASA biology space program, and consulting this article, it wasn't clear to me still exactly what I should cover in a talk such as this. So, I consulted my collaborators and colleagues about the studies we have been doing together and asked them if they could give me some insight and, also, supply me with a few slides to go over with you. After rummaging through slides, I came up with about 50 and decided that I would try to give you a taste of what is going on down in Tennessee. As this indicates, I will run through a lot of things in a superficial manner. There are areas of research in the lab that I will not cover at all. I will just throw out a few things to you and try to introduce you to the way we think, perverted as it may be to some of you, and, secondly, try to acquaint you with some of the dogma in the field of calcium modulated proteins.

As far as calcium's interaction with proteins, calcium binding proteins, like calmodulin fall into a class of proteins that are predominantly intracellular and reversibly bind calcium with dissociation constants in the micromolar to nanomolar range. Calcium regulation of these proteins appears to be due to localized increases in calcium concentrations in the cytoplasm. Our main interest is in purifying and characterizing the calcium receptors and to try to elucidate mechanistically how they are involved in cellular responses.

The early phase of this work I did when I was in Tom Vanaman's lab at Duke University. The project there was to characterize a non-muscle troponin C-like protein, which at that time no one had done, and to elucidate what activities this protein may have in the tissue of isolation. The protein was bovine brain calmodulin. It had regions of identity and homology with troponin C. The point here is that this data unequivocally demonstrated, in our opinion, that calmodulin is a member of a class of proteins that are both structurally and, as far as in vitro biochemical activity, functionally related.

As regard the in vitro biochemical activities of calmodulin, many of these are for regulation of nucleotides and, also, protein phosphylation. An area of research about which I will not be talking but is being done in our laboratory by Daniel Marshak is detailed studies of drug or dye interaction with calmodulin and related proteins.

So, trying to put the group's efforts into a cohesive organization, I organized what is going on as far as calmodulin and related proteins into three areas; first, purification and characterization of calcium targets and mainly calmodulin. Second, we are studying calcium modulated proteins and the molecular mechanism of action of these proteins, especially in terms of what they interact with in cell systems and how they go about exerting their action. Third, we and some people at Vanderbilt with whom we are collaborating are looking at the role of these calcium binding proteins in cell function. As I mentioned, I won't be talking about Dan's drug-protein interaction studies. Ray Zielinski from my lab, is looking at the general structure of calmodulin genes, but we will not discuss this work either.

As far as classic phylogenetics, I would like to go through now what is known about calmodulin.

First I will discuss a simplified phylogenetic tree of calmodulin. A lot of effort in the past has gone into studying calmodulin from vertebrates. The bottom line on that story is that it appears that most mammalian calmodulins, and possibly all vertebrate, calmodulins are potentially identical in sequence. A number of detailed studies have even been done on calmodulins from reptiles and amphibians and they are, also, very similar. Further down the tree into the area of insects and related species, and the plants, and fungi, we are concentrating our studies on a number of plant species. We have the nearly complete amino acid sequence of spinach calmodulin and have compared the amino acid sequence of barley calmodulin to it. Dr. Cormier is collaborating with Dr. Vanaman on mung bean and peanut, I believe, is the other species they are looking at.

It looks like this branch of the tree is falling into place. Further down the tree we are examining Chlamydomonas and are studying, in collaboration with Margaret Clark at Albert Einstein, Dictyostelium. In the amino acid sequence we determined for bovine brain calmodulin, there are definite sequence differences among higher plants, invertebrates, Tetrahymena and all the vertebrate sequences that have been done both in our laboratory and other laboratories. I don't want to go into Dr. Kretsinger's EF-Hand model, but we can identify in the sequence the helical regions surrounding proposed calcium binding loops. In my opinion Bob's EF-Hand hypothesis is probably one of the most significant contributions to this field in the last 30 years.

Comparative biochemical studies give information as to what calmodulin might be doing in different types of cells. The bottom line of this and some of the other data is that oftentimes the proof of the pudding is in the chemistry, and secondly, the more we learn the less we know. First, an example of how the method of isolation can result in apparent alterations of the properties of calmodulins. Dr. Burgess has done a very careful analysis of heat treatment of calmodulins. He has taken different species of calmodulins and subjected them to controlled heat treatment over periods of time and analyzed them on SDS gels with coomassie blue stain. This is gel A and this is gel B. Gel A is run in the presence of excess chelator. Gel B is run in the presence of excess calcium. Bob Kretsinger would have us believe that the difference in mobility here is due to differences in calcium occupancy state of these proteins. We find with increasing time of heat treatment, an appearance of multiple polypeptides above the main band. If you take this out further you can easily generate these to where they are of virtually equal intensity. We have looked at these heated samples in isoelectric focusing gels and they, also, show two IEF species. The basis of this structural change that occurs in this protein that is assumed to be heat stable we do not know. That is being pursued in the laboratory.

A related case study is that there are two proteins in Punctulata eggs that appear to be bona fide calmodulins but they differ in some of their detailed properties, such as the amount of histidine present. The point here is that these proteins appear to be calmodulins, and they appear to be iso-types. These are both isolated from sea urchins eggs. This is work done by Billy Burgess when he was in Bob Kretsinger's lab, and he is continuing these studies on characterization of these apparent isotypes.

The next example is from results that Michael Schleicher in my laboratory has obtained on barley calmodulin. The point here is that barley is thought to be unusual because it apparently lacks trimethyllysine, accordingly to the literature, and the rest of the composition appears to be quite dissimilar, including the apparent presence of five prolines. However, Michael's results summarized in this composition, which so far has been confirmed by direct sequence analysis of isolated peptides, indicate that, in fact, barley calmodulin is very similar, if not identical, to spinach calmodulin. I did not point out earlier that most of the plant calmodulins that have been carefully characterized appear to have one cysteine present in the sequence. It hasn't been determined unequivocally yet, but there is also cysteine-like material detected in barley.

Michael also isolated a barley calmodulin-like protein, and, as you can see, many of the differences in the published values and Michael's barley calmodulin preparation can be accounted for by the contamination of the purified calmodulin by this calmodulin-like protein. This calmodulin-like protein does not activate phosphodiesterase. It does not cross react with anti-spinach calmodulin or anti-vertebrate calmodulin antiserum.

Another case that apparently was quite different was _Dictyostelium_. Recently, in collaboration with Margaret Clark, we found that this calmodulin is contaminated by smaller peptides. This slide shows you the more recent _Dictyostelium_ composition, and it appears that it really does lack trimethyllysine but is not as different as previously thought. So, there are cases where you really do lack trimethyllysine.

Some more data by Michael Schleicher and Joseph Zendegni include comparisons of compositions of barley, corn, _Chlamydomonas_ and _Dictyostelium_. These studies show that in the higher plants the calmodulins are very homologous and, based on limited sequence analysis, it is potentially likely that they are all identical amino acid sequences. As you get further down the phylogenetic tree into the regions of _Dictyostelium_ and _Chlamydomonas_, it appears that both of these lack trimethyllysine. These will activate vertebrate phosphodiesterase. Coming back to the phylogenetic tree, we believe that the work on _Dictyostelium_ will give us insight into a major evolutionary branch of calmodulin. I did not address the work that Dr. Zielenski is doing on silkworm. It would be very interesting to obtain more information on this branch. As far as the plant species, I think between the work of Cormier, Vanaman and our laboratory we will have a very clear story of this branch of the tree. The current view is that calmodulin is really more highly conserved than we would have thought about one year ago.

What I would like to do next is skim through some data on binding proteins and give you some data using our gel overlay procedure. There are a lot of gel overlay procedures being published using calmodulin and other calcium binding proteins. We have spent three years addressing the question of how can we get a reproducible procedure that gives agreement, where possible, with studies on purified proteins in solution. These studies fall into our research area of calmodulin interactions and molecular mechanisms of action of these proteins. Our guidelines which we believe we have, after three years of work, been able to fulfill are shown here. This overlay procedure is generally applicable to most calcium modulated proteins we have examined. We are using it to elucidate the mechanism of action of what appear to be tissue specific calcium modulated proteins, such as S100, as well as more universal proteins, such as calmodulin. What I would like to do is show you a few examples and, also, propose a working hypothesis. To demonstrate the method, we can analyze a Coomassie blue stained gel and an autoradiogram using iodocalmodulin overlay, molecular weight standards and dilutions of myosin light chain kinase, a known calmodulin regulated protein. It is our philosophy that the more unequivocal data as far as calmodulin's involvement in physiological function indicate that myosin light chain kinase and phosphorylase kinase are two rigorous precedents that are good to use.

If you analyze a Coomassie blue stain and autoradiogram of the same type of gel, you get discrete bands of binding proteins when you have crude fractions that have a number of proteins in them. In fact, some of these binding proteins are actually very minor Coomassie blue staining bands. When you do the overlay in the presence of excess magnesium instead of calcium, you don't get the interaction.

Now, I want to show you two sets of gels of basically the same samples. This shows calmodulin interactions with purified proteins and crude fractions. Some of these proteins bind calmodulin in the presence of excess calcium and the presence of excess chelator or only in the presence of calcium. You get binding to many membranes or particulate proteins in the presence of excess chelator. The samples shown here are crude ammonium sulfate fractions, skeletal muscle phosphorylase kinase, smooth muscle light chain kinase, phosphorylase from skeletal muscle and myosin heavy chain. As you can see, these proteins bind iodocalmodulin only in the presence of calcium. In the presence of excess chelator these proteins do not interact with calmodulin.

DR. ROUX: After they bind the protein they migrate differently on the SDS gel, is that what you are saying?

DR. WATTERSON: No, I am glad you asked that. You dissolve the samples in SDS, and you run the gel. Then you go through a renaturation soak process. Most of these renaturation procedures were developed for enzymes by Lacks and coworker's at Brookhaven a number of years ago. These enzyme renaturation studies have been published in the Journal of Biological Chemistry and Analytical Biochemistry showing _in situ_ renaturation of enzymes. Sometimes they will apparently renature around the acrylamide matrix and you cannot elute them out, but you can do _in situ_ enzyme assays on them. In our overlay method, a well characterized iodocalmodulin which retains activity is put in an incubation mixture with the gel after a renaturation soak. The iodocalmodulin is added either in the presence of calcium, chelator or excess calmodulin. Then the gel is washed to remove excess label, fixed and exposed to film.

That is a very good point. I am sorry I did not go through that.

This is an iodotroponin C overlay with the same samples I showed you earlier. This shows that some of these calmodulin binding proteins will bind to troponin C. As you can see phosphorylase kinase is a troponin C binder. Similarly, some of the same proteins that bind iodotroponin C prepared in a particular way are, also, ones that unlabeled troponin C in excess can inhibit binding by iodocalmodulin. So, the modified protein binding is the same as the competition binding with the unmodified protein. We have done similar studies with unlabeled calmodulin. We showed that when iodocalmodulin is incubated with calmodulin there is no binding.

Myosin light chain kinase doesn't bind iodotroponin C and does bind calmodulin even in the presence of excess troponin C, whereas another physiological, binding protein, phosphorylase kinase, will bind troponin C and this binding is inhibited by excess troponin C. The next slide makes the point that the characteristics and the source of your iodinated and native protein is extremely important, I cannot emphasize that enough, as well as the particular protocol you are using. This shows some striking data of Michael Schleicher's using the same plant protein extract, and using either spinach iodocalmodulin in the overlay compared to using vertebrate iodocalmodulin in the overlay. After just a few hours of exposure, you can pick up the binding of spinach calmodulin to this plant binding protein and with the vertebrate iodocalmodulin you do not pick up the binding.

This is an old slide taken from a 1981 publication by Hertzberg and Gulula. It results from studies that Bernie Gulula and Linda Van Eldik and myself have been doing in collaboration for several years on the interaction of junction proteins with calmodulin. This is a crude liver plasma membrane fraction, Coomassie blue stained, and a partially purified liver gap junction protein and a lens membrane fraction. This shows you the corresponding radiogram from the overlay.

My point for this slide is that with the lens protein the binding by iodocalmodulin to this integral membrane protein appears to be calcium independent. It binds in excess calcium and it binds in excess chelator.

To summarize this section, there are three observations based on a lot of analyses that I would like to point out to you. First, there are a number of calmodulin-binding proteins that appear to bind calmodulin in a calcium-independent manner. Second, as I will show you in a minute, many of these calcium-independent binders appear to be associated with membrane fractions. Third, there are, also, a number of TnC binders. My point here is that group 1 and group 3 appear to overlap. Based on these observations we have a working hypothesis in the lab that calmodulin may use different structural domains in order to affect a differential subcellular localization. There are two corollaries of this model. One is a positive one as far as mechanisms of regulation; you could eliminate one diffusion limited step in calcium responses. There are two diffusion limited, or potentially diffusion-limited, steps in calcium responses calcium going to the calcium binding protein and then, with calmodulin and related calcium modulated proteins which have no enzymatic activity, that calcium-protein complex would have to diffuse to the binding protein.

Secondly, as a kind of a cautionary note, the example of phosphorylase kinase shows that calcium-dependent binding in the gel overlay method does not mean that in the supramolecular structure you have calcium-dependent interaction. In phosphorylase kinase, the binding to gamma subunit by the integral calmodulin subunit in phosphorylase kinase, is not calcium-dependent. That is, the calmodulin is not removable with excess chelator and exposure to chromatography. It appears to be very tightly bound and, in fact, it may be interacting with more than one polypeptide there.

As far as models of cell biology, potentially one could have one domain of calmodulin interacting with integral transmembrane proteins and another domain interacting with cytoplasmic proteins. This does not violate any of the known information about the proteins in vitro in detailed biochemical studies. So, it will be very interesting to pursue these interactions here in terms of what kind of domains are on the two different calmodulin binding proteins and what domains are used by calmodulin.

Finally, I would like to give you a little bit more data relating to this and keep pursuing the domain concept by quickly summarizing some work on transformed cells that mainly Linda Van Eldik has done. This has more to do with subcellular localization and looking at pathophysiological systems. A number of systems have been looked at, but in the transformed cell system calmodulin levels are increased compared to normal by three different criteria: quantitative gel electrophoresis, radioimmunoassay and activator activity.

Linda has shown that there is an apparent two to four-fold increase in transformed cells over normal cells in the amount of soluble calmodulin. In fact, in the studies I will show you now she actually showed that the total calmodulin levels are increased in all fractions, and the calmodulin molecule, as we have shown by isolation and detailed characterization of the protein, does not appear to be altered. Analysis of different subcellular fractions shows that there appears to be an increase in total calmodulin, not simply a redistribution of the calmodulin molecule. Linda did a detailed study with all marker enzymes and electron microscopy characterization of all the fractions and defined reproducible conditions for lysis of chick embryo fibroblasts and compared normal, transformed and, also, cells infected with a virus that has a deletion mutation in the transforming gene. So they replicate, but they don't transform. As you see here most of the calmodulin is in the S phase. Linda obtained a distribution pattern as protein versus relative specific activity for both normal and transformed cells. In the particulate fractions from transformed cells there is an amount of calmodulin equal or greater than, depending on the particular experiment done, the amount of calmodulin in the normal cell particulate fractions. However, when you detect calmodulin in these other subcellular fractions, even though it is reproducible, you cannot make any statements if the percent contamination of S-phase marker enzymes is equal to or greater than the percent of the calmodulin in the fractions. So, it is imperative you go in and show functional significance at that point. That is what Linda and Billy Burgess did, using overlay gels of cellular fractions: nuclear fraction, mitochondrial/lysosomal, P fraction or 100,000 G pellet and supernatant. These are enriched fractions. These are not homogeneous fractions. You can see that the membrane fraction has a number of calmodulin binding proteins, and there appears to be no major redistribution of the calmodulin binding proteins as a result of transformation. This definitely shows that there are calmodulin binding proteins present in these fractions. So, it lends some credence to the fact that you can reproducibly detect this small amount of calmodulin and suggests that further work should be done on purifying and characterizing these apparent membrane-bound calmodulin binding proteins.

The next slide shows you the fact that when you use excess chelator, as I showed you earlier, a lot of the proteins that bind calmodulin in the presence of chelator appear to be in membrane fractions of cells versus soluble fractions. The soluble binding proteins were inhibited by chelator. This shows you an overlay on the same fractions using iodotroponin C and, again you see that the TnC-binders and EDTA-binders are predominantly found in the P fraction. There are TnC-binders in other fractions, too.

Now, I would like to go through some data on immunochemistry of calmodulin.

This summarizes by a few examples the library of antisera we have generated. We have developed reproducible methods for the production of calmodulin antisera: 100 percent of the animals injected have produced high titer antiserum. We have characterized many of these in terms of their molecular and atomic requirements for interaction. In competition radioimmunoassay we can compare with the homologous iodoprotein how well things compete. As you see the antiserum will react with all the known calmodulins, including protozoan (s shown here by Tetrahymena calmodulin, a gift of Tom Vanaman), and plant calmodulins. These antisera are reacting, apparently with a portion of the molecule that is identical in sequence.

As far as this anti-vertebrate calmodulin serum (450), it reacts quite well with vertebrate CaM and poorly, if at all, with calmodulin from lower species. For this antiserum, 100 percent of reactivity is in a heptapeptide in the carboxy terminus of the molecule.

DR. ROUX: What is the difference between 449 and 450?

DR. WATTERSON: These are just two examples of the fact that the antisera have different immunoreactive sites. They were both produced by injection of vertebrate calmodulin. But their specificity as far as what they react with in the molecule is different. So, it allows you to generate a library of antisera to react with different portions of the molecule. Using plant calmodulin as the antigen you generate antisera that are specific to plant calmodulin. We have, also, developed methods for reproducibly making site-directed antisera by immobilizing synthetic peptides and using them as the immunogen and then characterizing their reactivity with a labeled, whole protein. This is relevant to the work of Burgess published in the Journal of Biological Chemistry in the A and B forms of CaM in eggs, two forms of calmodulin in the same cell. If you want to study the change in these after fertilization or during development you now have the tools to go in using these different site-directed antisera to differentially monitor the appearance and synthesis of one or the other forms.

The approach we used with the 450 antiserum, was primarily competition radioimmunoassay and 100 percent immunoreactivity was found in a cynanogen bromide fragment, and a tryptic fragment. We tested all the other ones, and they gave negative results. We went to using peptide synthesis and found that a unique heptapeptide had full immunoreactivity for this particular antiserum in competition radioimmunoassay. We, also, did synthetic substitution studies in which we looked at different side chain analogs. We also, compared a number of purified calmodulins as far as their immunoreactivity. From this we can explain now why no other domains within the calmodulin molecule react with this antiserum. This sequence is not found anywhere else in the internally homologous domains of the calmodulin molecule. We could, also, answer the question why doesn't it react with troponin C's. That is because they have these substitutions here. The lack of reactivity is confirmed by sequencing and the synthesis of these peptides and testing them. We can, also, go to the other calmodulin and make a strong statement about when we find differences in reactivity with 450, it is due to a single charge substitution in the side chain here because these are the areas of sequence from these other calmodulins. As far as the three-dimensional model based on Bob's EF hand model of what calmodulin should look like, we can now identify the portion of the molecule which this particular antiserum reacts with; towards the carboxy terminus. By having this and other antisera that react with different parts in the molecule we can do some studies on mapping the protein. We are using these same immunochemical approaches with other calcium modulated proteins and with calmodulin binding proteins and S100 binding proteins.

We can now compare on the same samples in a well-controlled study, activator activity and immunoreactivity using this particular 450 antiserum. We compared boiled and unboiled samples of phosphorylase kinase. We put the same sample in two different tubes, kept one at room temperature and heat treated one for a short period of time. We then tested for both activator activity and for immunoreactivity. There is a slight but really not too significant difference in immunoreactivity with the 450-type specific antiserum as far as immunoreactivity, indicating that that domain on the molecule is exposed. As far as activator activity of phosphodiesterase there is very little or any activation by the phoskinase without heat treatment, but upon heat treatment you release the calmodulin from its bound state, and it can then activate. That is an important concept. If I lost you, I will try to summarize it here. This and other studies which I don't have time to go through show that calmodulin's activator domain is not accessible when it is part of the phoskinase complex. It shows that its PDE-activator domain is accessible when it is bound to some other proteins and we can use site directed antiserum, such as the 450-type specific antiserum, to map domains and show that the various immunoreactive sites are exposed.

In conclusion, what this would indicate is that antisera directed against this region would be exceptionally useful in terms of biological studies because, so far, we have not found any supramolecular complex in which we can mask this particular immunoreactive site.

I will conclude by saying that I know I have gone through a lot of information here. I hope I have given you a taste of what we do down in Tennessee, and I would be glad to pursue any other discussions you would like to have.

Thank you.

DR. ROUX: Questions?

DR. THOMPSON: You told us about some of the intracellular and the membrane bound calmodulin binding proteins. I wonder if you would mind commenting on some recent data that suggests that at least in a couple of organisms like Paramecium and Tetrahymena there is an extracellular calmodulin-like protein? It seems to me that if that might be the case in other cells that it would certainly confuse people's cytochemical efforts to localize where calmodulin or calmodulin binding proteins would be, and it is curious to me whether there might even be a membrane-bound, an integral membrane protein that would have a calmodulin binding affinity towards the outside of the cell.

DR. WATTERSON: You said calmodulin-like protein, and you, also, said calmodulin binding. Do you mean the data with extracellular calmodulin binding proteins?

DR. THOMPSON: I think the data suggest that there is a calmodulin, an extracellular calmodulin, and I am wondering whether there may be a membrane bound calmodulin binding protein that might face the outside of the cell as well as one that faced the inside. Of course, the philosophy of those would be quite different depending on whether facing the inside or the outside.

DR. WATTERSON: I hope I made the point subtly that what is a calmodulin needs to be rigorously defined.

DR. THOMPSON: Yes, okay.

DR. WATTERSON: So, as far as extracellular calmodulins we still, in our laboratory, reserve the right to question whether they are looking at calmodulin, and whether they are looking at physiological phenomenology or not. I mean, of course, if you lyse a cell you are going to get calmodulin extracellularly. The question is whether calmodulin is present, physiologically, outside the cell. Calmodulin binding proteins may be extracellular in orientation. There are prokaryotic calmodulin binding proteins. That is quite a different story, okay? That doesn't mean that they are regulated by calmodulin necessarily. That is something that needs to be pursued.

I think our work and the work of other laboratories indicates that this is an exciting possibility as far as molecular pathology, and what I want to show you in this slide, if you will notice these binding proteins here, Dr. Schleicher in our laboratory has done a number of studies on purifying membrane proteins from bacteria that are calmodulin binding proteins by overlay criteria. He is trying to do some solution studies. This is difficult work because they are membrane proteins. But they are purified outer membrane proteins from bacteria and bona fide calmodulin will bind to them. Now, there may be calmodulin-like proteins, also, around. In fact, one has to ask the question why did mother nature make bacteria have calmodulin binding domains. Is this a biochemist's artifact or is this something real, and maybe it is involved in molecular pathology or maybe there is a calmodulin-like protein that is present in bacteria themselves, and that is a very legitimate question. That is something that needs to be answered, and people are looking at that.

DR. THOMPSON: But I am talking about eukaryotic cells in protozoa, and as I understand, it has virtually all the characteristics of calmodulin, including trimethyllysine.

DR. WATTERSON: As I just showed you, I don't think trimethyllysine necessarily means it is calmodulin. The proof of the pudding is in the chemistry. So, I strongly question that, and I strongly question the data.

DR. THOMPSON: You don't think then that it has yet been proved, at least, to be an extracellular calmodulin?

DR. WATTERSON: To my satisfaction, correct, yes. It has yet to be proved. I am not saying one cannot exist. We and others are very interested in the possibility of whether calmodulin or related proteins are extracellular.

DR. ROSS: One question I hoped to have answered here was whether or not bacteria did, indeed, have calmodulin or something like it. Do I understand that there really is not an answer to that question? I mean from what I have read everybody starts with the nucleated cell.

DR. WATTERSON: In other words a prokaryotic cell, prokaryotic bacteria is what you are asking. I think it is very doubtful with the data right now that there is a prokaryotic "bona fide" calmodulin. There may be. We have not found it. A lot of people have looked for it. It is one of those things that you don't publish, if you do a rigorous study, and you don't find it.

Bob has published data in which he has looked for it in a couple of prokaryotic bacteria, and he has not found it. We have used activator activity, immunochemistry methods with a battery of antisera, one, for example, antiserum that reacts with all calmodulin, bona fide calmodulins, and don't detect much of anything. If we do, we will have to go in and prove that it is calmodulin. As you can see as you get down towards the bottom of the phylogenetic tree there is a lot of jumping around that is going on as far as the structure and potential function, too. I think Milt might address this point as far as the function. It gets very interesting down around plants and algae.

DR. ROSS: If I could elaborate why I asked the question, I would be very much interested in streptomycin and how it acts, and I read a couple of recent reviews lately in which it was indicated by the author who had done some work on it that the lethal step appeared to be some calcium binding material inside the cell, and --

DR. WATTERSON: There are calcium binding proteins in prokaryotic cells. There have been crystal structures done on them.

DR. ROSS: But it isn't necessarily calmodulin?

DR. WATTERSON: Yes, you know, one of the points in the first slide which I went over a little too fast, I think, is that calmodulin is just one example of a particular class of calcium binding proteins. There are all kinds of classes of calcium binding proteins, as well as what people are now calling calcium modulated proteins. There are also ones that appear to be tissue specific. Troponin C is a calcium modulated protein, but it is only found in striated muscle. All the data indicates that that is where it is found, and calmodulin appears to be found in most eukaryotic cells examined. Calmodulin-like proteins and other factors bind to phenothiazines and go through calmodulin purification protocols, but when you get down to where you really pull these apart they don't have activator activity. They may or may not have trimethyllysine. They don't react with antisera. Those are around. Nobody knows what they are doing as far as we know. There may be similar things with prokaryotes.

DR. KRIKORIAN: Do you know of anyone who has identified mutant strains of yeast or Dictyostelium that don't have these binding, calcium binding proteins or have sufficient variations so that you have got yet another kind of evidence for your domain?

DR. WATTERSON: Not yet.

DR. KRIKORIAN: You can dig out an awful lot of mutants in yeast, of course.

DR. WATTERSON: Yes.

DR. KRIKORIAN: Plus it is a eukaryote.

DR. WATTERSON: Yes. There are other mutant systems for calmodulin. In the I-strain of mice, which is analogous to McArdle's syndrome in which you have calcium regulated phosphorylase kinase missing in striated muscle, there is a dimunition in the amount of calmodulin in whole muscle cell extracts that is on the same order of magnitude as is calculated to be in the phoskinase complex. So, you have a genetic system there you can also look at, and people are doing that.

Secondarily, as far as other mutants, I believe there are a couple of reports about phenothiazine resistant mutants that the authors are postulating are calmodulin deficient. Do you remember the species, Ray? Volvox maybe. I think there was something that came out on that in the European Journal of Biochmistry (1982).

DR. CHAFOULEAS: Those mutants for stelazine and the other phenothiazines usually are the calmodulin binding proteins. They are deficient in one specific calmodulin binding protein, but the mutation doesn't seem to be on calmodulin.

DR. WATTERSON: I see.

DR. KRIKORIAN: One of the reasons why I asked that is that I remember and I have done some work on this myself, is that most fungi can survive with virtually no calcium in the culture media. One would wonder in the case of a Neurospora situation whether there is a calcium-binding protein.

DR. WATTERSON: Calcium in the extracellular medium doesn't necessarily reflect what is going on inside the cell.

DR. KRIKORIAN: Exactly.

DR. WATTERSON: Some cell types are more dependent on extracellular calcium. In fact, we got into the transformation story back in the seventies because of the simple observation that transformed cells grow in calcium deficient media, whereas the same normal cells couldn't. So, you can alter the extracellular requirement and still not necessarily have extracellular calmodulin.

DR. ROUX: Another approach to immunological site specificity would be the monoclonal antibody approach. Are you looking at that in your lab or do you know if this has been worked?

DR. WATTERSON: We are looking at it in our laboratory. Actually with the synthetic peptides, the need for monoclonal antibodies isn't there. So, it is not a high priority item, but we have been playing around with characterizing them and trying to generate, specifically, more IgG production by monoclonals. I mean it is just as much work there. With monoclonals you still have to go in and characterize what it is reacting with. So, it is in fact less work to make an antigen and do your selecting at that point. It is also cheaper. Money seems to be a factor these days.

DR. REED: Do you have calmodulin binding in a calcium independent fashion now with some proteins and membranes? That sort of removes calcium from being a regulatory part of that.

DR. WATTERSON: There are two phenomena which people tend to forget. There is protein-protein interaction, and there is protein stimulation or effector activity. So calmodulin can bind to a protein in a calcium independent manner but still have a calcium dependent conformational change that regulates activity.

DR. REED: So, you are not saying it is independent of calcium for activity, just protein binding.

DR. WATTERSON: Yes. In addition, using chemical modification we have some suggestive data that we might be able to actually dissociate protein binding from activator activity. So that is two different questions.

DR. TSIEN: Do you find or do you ever look at the possible modulation of a calcium affinity by a different effector protein or other proteins which can bind to and make tertiary and quaternary complexes? The reason I ask is that all this isolation of calmodulin everywhere, I wonder whether it really means that the calcium changes inside the cell are really triggering anything. Could it not be that calcium is just needed there at a static level in the way that the pH has to be a correct level; the calcium calmodulin happens to be sensitive to that and that these things get turned on by some other activation, but the cell doesn't use calcium.

DR. WATTERSON: There is a basic assumption which you have made there which I would like to address, okay? That is calcium homeostasis on a molecular level is not simply an equation of calcium plus calmodulin. There are these other tissue specific calcium binding proteins. For example, troponin C and, S100 appear to be only in certain cell types. How do these different calcium modulated proteins interact with each other? There may be tissue specific types of calcium modulated proteins. There may be ones that are found only in certain subcellular organelles. There may be ones that are found only in certain species. There may be ones that are found only at certain stages of differentiation. So, now, one could think what calmodulin is doing in the cell is the question and whether in some cells it is mainly the kind of on/off switch and other places it is simply kind of like an accelerator that allows you to speed up things or slow them down or furnishes a loop mechanism around the metabolic block by the tissue specific form; those are all open questions as far as I am concerned. So, it is a legit question, but I would caution against just trying to extrapolate from just calmodulin chemistry and biochemistry that that is all that is going on.

As far as your first question about detailed studies and how they interact with other ones, I think Bob has indicated this before. They have done some modeling studies which are interesting, and he could probably better address the studies on calcium binding of calmodulin in a complex. Those are very, very important questions that we are not looking at, but other people are.

DR. ROUX: Last question before lunch? Guy?

DR. THOMPSON: I think a related question to this one is it seems like there is so much calmodulin in some cells, do we really need that number of molecules to go around to all the functions or is there seemingly an excess of what we can account for?

DR. WATTERSON: There could be an excess. We don't know. We saw these numerous binding proteins and different fractions I showed earlier. How much of those are there? What is their relative affinity? We don't know. The overlay technique is not a quantitative technique. So what needs to be done, in my opinion, is to purify and characterize them, look at the relative affinities, look at how much of them there are under different physiological states and then you can start doing a number of things to answer that question. There is really one part of the equation missing, and nobody has gotten it as far as I know. It is in a particular cell type to rigorously characterize how many calmodulin proteins are there, what their activities are; how much of them is there; is there really free calmodulin floating around? Now, under biochemical cytology techniques we get this large amount in the S phase, but inside the cell we don't know that that is free, and a lot of it may be bound up in those other fractions, and the conditions we use just don't keep it there; the same for the immunocytochemistry. I think someone earlier was making the point about fixation and whether there was a change or not. So, it is an open question and I don't know the answer to it. But, there are relatively high amounts of it.

DR. CORMIER: I might comment on part of that question. It has been shown by a number of investigators that as you increase the calmodulin concentration you decrease the amount of calcium required to activate a particular system. Mass action would dictate that. So, part of the reason, depending on the cell type with the amount of calmodulin there, might be related to the amount of calcium generated in response to a stimulus.

DR. THOMPSON: Can you vary just the amount of of calcium available on, say, the outside of the cell, and thus tend to vary the amount of calmodulin over a long period of time?

DR. CORMIER: I would doubt it.

DR. WATTERSON: We are looking at that and it is tough to try to do chemically defined media culture and then adapt some cells to defined media and very low calcium. Technically it is extremely difficult and may be a futile effort. I don't know. For some of these cells, it is doubtful that you can even adapt them to calcium.

DR. ROUX: Thank you all for an excellent morning session. Let us take lunch.

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DR. ROUX: Okay, we are ready to begin the afternoon session. The first speaker this afternoon will be DR. MILTON Cormier from the University of Georgia who will talk to us about the role of calcium and calmodulin in plant cell regulation.

DR. CORMIER: In line with the goals of the conference, I thought I would spend a few minutes making a few general comments about calmodulin, especially plant calmodulin, at the risk of overlapping with some of Marty's comments and then tell you a bit about what we have been doing recently on the role of calmodulin in certain plant systems.

In animal systems, at least, there is a vast literature, as you well know, on the effects of calcium on various physiological phenomena, and that goes all the way back a century ago to the time of Ringer and his colleagues who worked on muscle contractions. There is a very, very long list of those kinds of examples, but in the plant field the kinds of effects that calcium has on various physiological phenomena in plants is of fairly recent literature. I show you a slide which illustrates some of that. This is by no means complete. I just want to throw up a few examples of what we know about the role of calcium. You will hear a number of others here, I am sure, but one of the first examples of a calcium effect on a physiological system in plants was made in 1959, on the chloroplast rotation in Mougeotia in which the chloroplast rotation phenomena is a calcium dependent process, and then Stanley Roux and others have been working on phytochrome mediated effects of calcium transport, and there are some effects on Nitella cells which are dependent on calcium, cytoplasmic streaming being one of them, and I will say a little bit more about that perhaps later on.

Now, it wasn't until the discovery of calcium-binding proteins in animal systems that we had any sort of handle on a molecular approach to the understanding of calcium effects in those systems, and similarly it wasn't until the isolation of calcium binding proteins, such as calmodulin from a variety of plant and fungal systems that we began to have an approach to the molecular understanding of the effects of calcium in plant cells.

Now, let me throw out a little, as Marty says, a little dogma to you, and this dogma is based primarily on some concepts generated by Bob Kretsinger and earlier on the second messenger hypothesis by Rasmussen, but basically this is the way we think about the effects of calcium and calmodulin in animal and plant cells, that is to say there is a receptor-mediated effect on the intracellular concentrations of calcium in response to some stimulus.

The intracellular calcium levels in animal cells have been looked at by a variety of techniques, as many of you know. The bioluminescent protein which I am more familiar with perhaps, aequorin, has been used by many investigators. It is a calcium binding protein that falls into this super family of calcium binding proteins which binds calcium and emits a photon of light in response to some chemistry and the generation of an electronic excited state.

Now, that has been a very useful probe. Tsien's compounds that he will discuss later are, also, extremely interesting ones; the arsenazo dyes and a number of techniques have been used to probe intracellular levels of calcium, and in general most of those techniques agree, and it tells us that the levels of intracellular calcium in the animal cells that have been looked at are on the order of 1/10 micromolar or less.

Now, there has been a recent experiment reported a few months ago by Williamson and Ashley of Oxford who injected Nitella, a green alga, with the aequorin and looked at the effect of stimulation on the light that was emitted in the cytoplasm and it was interesting to us because we were trying to do the same experiment and in fact, we have repeated parts of their experiment and that is that if you do inject aequorin into these cells, what you find is that there is an initial amount of light produced and then the light subsides. You don't see any more light, and you come back 12 to 14 hours later, and find that 90 percent of the aequorin is still there that you injected 12 to 14 hours earlier. What that means to me is that the levels of intracellular calcium in those cells are very low, certainly less than 1/10 micromolar and in that regard then plant cells are very much like, at least *Nitella* cells, are very much like animal cells in the sense that they do keep their calcium levels very low. Now, upon stimulation, the calcium levels presumably rise as shown by all of these other techniques I have mentioned and will bind to calmodulin presumably activating a series of enzymes depending upon the cell type, one or more such enzymes leading to a cellular response, and that is the dogma and the way that this is currently being viewed.

Figure 1 is, again, some of the information that Marty presented, just shown a little bit differently and that is that there are a variety of enzymes that calmodulin is supposedly regulating *in vivo*. In most cases we know with certainly it regulates these enzymes *in vitro* and in some cases there is reasonably good evidence that *in vivo* regulation occurs, but when calcium binds to calmodulin the available literature says that one or more calcium binding sites, of which there are four, are occupied to produce a distinct conformational state of calmodulin which then binds to one of these enzymes and activates it.

Now, presumably there are a variety of cellular processes that could be regulated by calmodulin, such as motility and cytoskeletal function, glycogen metabolism, cyclic nucleotide metabolism, calcium metabolism itself, with the calcium transport ATPase, and I will try to provide you some evidence that suggests that perhaps photosynthesis could be added, and there are others to be added to this list.

Figure 2 is a model of calmodulin. The sequence, of course, was derived from Watterson's work with Tom Vanaman and what I did is go through the literature and pull out what was known about the replacement that occurs in invertebrate, plant, and protozoan calmodulin replacements in the vertebrate calmodulin sequence. There are a couple of things I want to point out here, and that is that at least the first half of the molecule has been very highly conserved, as you can see, and most of the replacements take place around domains 3 and 4 of the molecule and that may have some significance biologically as I will indicate later on for plant calmodulin where some differences in activity are noted.

The other thing I want to point out here is that an ex-student of mine, Harry Charbonneau who is now working in Tom Vanaman's lab and I have been collaborating on trying to pin down the position of the cysteine residue that occurs in plant calmodulin. We can generate two peptides cleaved specifically at position 26 or we can generate the tryptic peptide beginning at position 30. The n-terminal portion of that peptide has been sequenced, and the cysteine residue is placed in position 26 replacing threonine in vertebrate calmodulin.

Now, the other interesting thing about that is it is modified in some way. It does not exist as free cysteine. One has to reduce it with dithioerythritol prior to titrating it with sulfhydryl reagents and one can then make a number of replacements, a number of modifications of that cysteine residue, such as carboxymethyl derivatives and so forth. When you do that and you then look at the activity of that protein and look at its ability to activate phosphodiesterase, for example, or its ability to activate the plant enzyme I am going to talk about later, NAD kinase, you cannot see any differences between the modified ones and the native form or the reduced form, suggesting that at least that replacement doesn't seem to have any biological significance thus far.

I want to spend the rest of the time talking to you about an enzyme that we came across a number of years ago that became interesting to us, and that is the enzyme NAD kinase in plants. NAD kinase is the enzyme, of course, that takes NAD, the cofactor and ATP and makes the phosphorylated form of the coenzyme NADP out of it.

We found that early on this was a calcium-sensitive enzyme and we became interested in an observation made in Japan by Muto and Myachi who found that there was a protein activator of that NAD kinase. We isolated that activator and found that it was identical with calmodulin and once you purify the NAD kinase as we have now done to apparent homogeneity, one can show that calcium and calmodulin are absolutely required for the activity of this enzyme. Also, the calmodulin inhibitor, trifluoperazine will, also, inhibit its activity in vitro.

Now, another feature of that enzyme is that if you compare its dependence on calcium concentration with vertebrate calmodulin and its activation of phosphodiesterase which is shown in one of these curves, the other one is NAD kinase being activated by plant calmodulin. One can readily deduce that they both respond, both plant calmodulin activation of a plant enzyme and vertebrate, calmodulin activation of a vertebrate enzyme respond similarly to calcium concentrations in the micromolar range. This is, also, true if one looks at the enhancement of tyrosine fluorescence that one gets when calcium binds to calmodulin. There is a big, I say, big two-and-one-half-fold increase in tyrosine fluorescence. Again one sees essentially identical calcium profiles.

Here is a titration of NAD kinase by plant calmodulin, that is calmodulin isolated from mung beans and notice that the amount required for half saturation lies roughly about .5 nanograms per ml in the assay. Again, if one uses the same solutions to look at the titration of calcium ATPase derived from erythrocyte membranes this is what you see.

Now, it turns out although I don't have all the data here to show you, and you will have to take my word for it, that if you compare plant calmodulin versus vertebrate calmodulin in the activation of the calcium ATPase they are the same. They fall on top of this curve. Phosphoioesterase is somewhere in the middle so that, again, if you titrate phosphodiesterase with either plant calmodulin or animal calmodulin, you get the same effect.

On the other hand, if you go back and use vertebrate calmodulin to try to titrate NAD kinase you find that the curve has shifted about 10-or-20-fold. So what I am saying is that the plant enzyme can distinguish between plant calmodulin and animal calmodulin or at least vertebrate calmodulin or invertebrate calmodulin for that matter, whereas some of the animal enzymes cannot distinguish those two and that is why I said that perhaps some of the substitutions in domains 3 and 4 might be important in this kind of recognition that we were seeing.

During the purification of NAD knase, one of the methods that we use is to put the crude extract or partially purified enzyme over an immobilized calmodulin column. A lot of people have done this and shown the same thing with other enzymes earlier on. This is a calmodulin sepharose column. What happens is most of the protein goes through. If you hit it with salt in a calcium-containing buffer, okay. That is very important. The NAD kinase activity sticks to the column in the presence of calcium because presumably the enzyme is binding to the calmodulin on the column, and then when you remove calcium by putting in EGTA then the NAD kinase activity comes off. That experiment suggests, as has been suggested for a number of animal enzymes, that the calcium calmodulin complex is simply associated with NAD kinase to form the complex, converting the inactive enzyme into an active one.

Now, at this point we have shown that at least in vitro the enzyme NAD kinase depends absolutely on calcium and calmodulin for activity and that is nice except that one would always like to know whether that has any physiological relevance. This is a problem that we have been concentrating on in the last few months, and I will try to address some of those questions as we go along. Whether or not NAD kinase regulation occurs in vivo, one can get some idea of this from the literature. It was shown a number of years ago by these investigators that if you shine light on a green leaf, or on Chlorella that there is an increase in the NADP/NAD ratio in those tissues, suggesting that there is a light-induced regulation occurring and with the assumption being made here in our case that it is occurring at the NAD kinase level. We have, also, shown during the purification of this enzyme that at least 90 percent of all the NAD kinase that we can find depends on calcium and calmodulin for activity. We don't see any other species of enzyme existing in extracts of plants, at least in our hands.

There is an excess of calmodulin over NAD kinase in extracts of pea seedlings which is what we have been working with, at least a 10-fold molar excess and the light dependent conversion of NAD to NADP is too fast to suggest de novo synthesis of NAD kinase because it occurs in over a second time scale. Now, if we want to throw in some more dogma, we can go a little further in the case of plant cells and suggest that there is a receptor mediated change in the calcium levels in some part of the plant cell by light which then activates the NAD kinase through the formation of a calcium calmodulin complex and that, simplistic view would then explain why it is when you shine a light on a green leaf you see an increase in the NADP/NAD ratio in those tissues. Since this is a light-induced phenomenon, the next question that we wanted to try to address was whether or not this occurred in the chloroplast or in some other part of the cell, and I might add that Harry Jarrett in my lab has done quite a bit of work on that over the last six months, and I will tell you a little bit about his work as we proceed.

Again, if you look at the literature you find that Muto and Miyachi and their colleagues in Japan recently showed that about 90 percent of the NAD kinase is localized in the chloroplast of higher plants, at least in five out of six species that they looked at, and we have repeated that experiment with chloroplasts isolated from peas and again showed that very high levels of the NAD kinase, most of it was localized in the chloroplasts.

These same people have shown that there is a light-induced conversion of NAD to NADP in isolated chloroplasts, and in addition to that they have made another very interesting observation recently and that is that if you shine light on isolated chloroplasts there is a very rapid uptake of calcium 45 into the chloroplasts and all of that taken together with our own data that the NAD kinase is regulated by calcium and calmodulin suggest perhaps this is occurring in the chloroplasts. What we did, was to say, "Okay, if that is going on, then the calmodulin has got to be in the chloroplasts," and this is one of the things that Jarrett has worked on in my lab recently.

We isolated chloroplasts from green tissue, from pea seedlings, and tried to take the precautions necessary to ensure intacness, that is to say using enzyme markers specific for chloroplasts, specific for organelles, specific for the cyptoplasm and so forth and also, to assure ourselves that we were not trapping calmodulin simply through the purification of chloroplasts, and that we did by throwing in massive amounts of radioisotopically labeled calmodulin in the very beginning, using iodine-125 calmodulin. We, also, used C^{-14} calmodulin, and then homogenized the tissue and carried through the chloroplast isolation and showed that of the calmodulin or calmodulin-like protein we could isolate, that the contamination levels would have been less than .1 percent based on the isotope we found. So, we don't believe that we were carrying along an impurity.

The other thing we did was to show that whatever it is we were looking at came from within the chloroplast and not on the outside. This we did by a series of trypsin digestion experiments that show that if you take a chloroplast preparation under iso-osmotic conditions and add it to NAD kinase assays, no activation was seen. But, as soon as you disrupt the chloroplasts under mild conditions by hyoptonic shock in water and then centrifuge the particulate material, the supernatant then contained an activating factor which is the data that I am going to show you here, and trypsin would digest this factor and eliminate it, but would not do so with intact chloroplasts. This would only happen if you disrupt the chloroplasts and then treat with trypsin, but what this experiment shows is that if you look at NAD kinase activity, and in the presence of purified mung bean calmodulin, you can easily see the calcium effect as you would expect, and the trifluoperazine inhibition of that activation.

Similarly in the crude extracts of pea seedlings you see the same thing and, also, in this chloroplastic activator we see the same thing, calcium stimulation and a trifluoperazine inhibition of that activation. It turns out that this protein or this activator contains many properties in common with calmodulin. For example, and I probably won't have time to show you all the data, but on SDS gels it comigrates with mung bean calmodulin and it shows a calcium dependent mobility effect on the gels as you see with calmodulin in general. It is an acidic protein. It is heat stable. It, also, binds to phenothiazine affinity columns, such as fluphenazine sepharose for example. We know that some of these phenothiazines bind to calmodulin in the presence of calcium and when that happens you shut down calmodulin function. You can make columns out of this material and use it for purification of calmodulin. If you put through a chloroplastic extract one can show that indeed the chloroplastic activator sticks to the fluphenazine in the presence of calcium. As soon as you remove the calcium, then the chloroplastic activator comes off. So, it says that there is fluphenazine binding site which is calcium dependent, just as you would see with calmodulin itself. Again, if you take the chloroplastic activator and you look at the activation of NAD kinase as a function of calmodulin concentration, and adjust the concentration of the chloroplastic activator so that it is at least within a factor of two of the mung bean protein and do the titration, you see that the curves are very similar, again, suggesting that the chloroplastic activator is behaving like calmodulin. We suggest that it is calmodulin, but we cannot prove it because we cannot get enough protein. I mean it is hard to isolate enough purified, intact chloroplasts to pull out enough protein to do classical chemistry with. So, we were kind of stuck there for a while.

But if you take all of the data that I have referred, it suggests that calmodulin could be viewed as having an effect on the rate of photosynthesis because after all we are generating NADP here by this mechanism, and that is one of the next things we wanted to look at.

We repeated these light-induced NAD to NADP conversions in protoplasts. You can show that, indeed, if you shine the light on these protoplasts the NADP levels go up; NAD levels go down and so forth and that if you throw into this gamische some of the inhibitors of calmodulin function, such as chlorpromazine we see an inhibition of the conversion at about 50 micromolar. If you throw in chlorpromazine sulfoxide which does not interfere with in vitro calmodulin function you don't see a significant effect. We have also used the W7 and W5 pairs, which I think are better comparisons because those two compounds differ by only a chlorine atom in the naphthalene ring and the hydrophobic character of those two compounds are very similar, within 10 to 15 percent as measured by partition coefficients. For that reason we used those compounds as well, and again showed that you could inhibit this conversion of NAD to NADP by W7 but not with W5 at about 50 to 70 micromolar each.

We measure oxygen evolution from these protoplasts that we made, and this was done with the help of Clanton Black in our department. If you shine light on these protoplasts, oxygen evolution begins. If you begin throwing in trifluoperazine or chlorpromazine at 50 micromolar we see that eventually you can inhibit this oxygen evolution. Chlorpromazine sulfoxide at 50 micromolar has a small effect, but it is not nearly as pronounced as chlorpromazine at the 50 micromolar level, and we have, also, done these experiments recently with W7 and W5; again with W7 at about 50 to 70 micromolar you see almost complete inhibition of oxygen evolution, but with W5 you see essentially no effect. So, again, suggesting that there is effect perhaps on calmodulin, but here again I cannot emphasize enough that these compounds are amphipathic cationic detergents, especially the phenothiazines. They bind to various membranes with effects on membrane structure and function.

That is why I prefer, if you are going to use these probes, the W7, W5 comparison because at least you have compounds of roughly equal hydrophobicity to compare it to, and if you see an effect with those, with W7 and not with W5, you feel a little bit more comfortable anyway. But again, I caution anyone who uses these compounds. The next figure (Figure 3) gives the concentration dependency of trifluoperazine on that oxygen evolution that I was showing you.

The interesting thing about this, of course, is that the amount required for 50 percent inhibition of our calmodulin dependent phosphodiesterase activation is about the same as you see here for oxygen evolution. Whether that means anything or not, again, I don't know. But if you take all the data that I have shown you together we can postulate some more dogma: as shown in Figure 4 I would like to suggest perhaps that within the chloroplast all of this is occurring. As we have already shown and others have shown, NAD kinase certainly exists in the chloroplast. There is a light dependent calcium influx that I mentioned in isolated chloroplasts. We have shown that calmodulin or something very similar to it exists in chloroplasts and therefore suggests that the light stimulation of this conversion of NAD to NADP is regulated through this calmodulin pathway. If that is correct, then of course calmodulin would be considered a regulatory component of the photosynthetic apparatus because NADP is the primary acceptor of the reducing power of water through photosystems 2 and 1 and the generation of NADP then is one of the components required for the initiation of the Calvin cycle.

I think I will stop right there.

DR. HEPLER: I am interested in if you have more detail on the light stimulation of calcium. Do you know the action spectrum for that? Is it the same as photosynthesis?

DR. CORMIER: Yes, some of these things we really would like to address. No, that information is not available. I think more quantitative data is needed on those experiments as well.

DR. HEPLER: But you have not done an action spectrum?

DR. CORMIER: No, whether it is phytochrome or anything like that?

DR. HEPLER: Yes.

DR. CORMIER: I proposed that in my grant proposal.

DR. CLELAND: Does the chlorpromazine reduce the endogenous NADP, NAD ratio in the dark? In other words, if you start out with a certain level in the dark, does it reduce that?

DR. CORMIER: It doesn't reduce it over the time period that we were looking at it.

DR. CLELAND: Because I noticed that the oxygen evolution when you turn on the light shows no lag suggesting that -

DR. CORMIER: There is a small lag. I don't know whether you can pick it up.

DR. CLELAND: How rapidly does the light change the NADP, NAD ratio in the chloroplasts?

DR. CORMIER: That happens over about the same time scale as it occurs in the green leaf which is, you know, in a matter of a minute or two it is complete.

DR. ROUX: There is some indication in the literature although I don't really believe a lot of that, that the concentration of calcium in the chloroplasts is reasonably high. I don't think the studies were really done that carefully.

DR. CORMIER: But you are talking about total calcium.

DR. ROUX: Correct.

DR. CORMIER: Okay.

DR. ROUX: I think an important part of this hypothesis would be to somehow show, if possible, that the free calcium concentration in the chloroplasts was micromolar because otherwise the calmodulin would always be activated and the light-induced calcium uptake really would not have much of an effect.

DR. CORMIER: Absolutely, but the chloroplast data on calcium is no different from whole cell data. I mean in other words there are millimolar levels of calcium there, but it is total calcium and so what we really would like to address is what the free calcium levels are.

DR. ROUX: What is the subcompartment of NAD kinase in the chloroplasts, do you have any idea?

DR. CORMIER: The concentration of NAD kinase?

DR. ROUX. The subcompartment, where in the --

DR. CORMIER: Oh, no,we have no idea. That is something else. We have no idea where the calmodulin is which is another thing we would like to address.

DR. KRETSINGER: Milt, I think you gave the numbers and I just could not hear them. What is the, in your fractionation procedure, what is the partition of calmodulin and calmodulin-like protein between the cytosol and the chloroplasts, what are the relative concentrations?

DR. CORMIER: Good point. I did not mention it, actually, but it turns out that you can only account for about 1 percent of the total calmodulin in the plant cell being derived from the chloroplast. In the chloroplast itself, if that activator truly is calmodulin, it would represent a concentration of about .2 micromolar in the chloroplast.

We know that the NAD kinase levels are significantly lower that that. So there is plenty enough calmodulin or chloroplastic activator in he chloroplast to do the job and then to have plenty left over.

DR. BANDURSKI; You said the cysteine residue was somehow modified. Is it a disulfide?

DR. CORMIER: No, it is certainly not a disulfide, and if it is modified with a low molecular weight group we have not been able to pick it up. It could be simply a difference in oxidation state which is, you know, reversible. Only after you reduce it with dithioerythritol can you get it to react with these SH reagents.

DR. ROUX: I would like to further ask questions on that curious sulfhydryl group there. You have published studies indicating that when plant calmodulin aggregates and it does have a higher tendency to aggregate than the animal calmodulin, according to your studies -- that this reduces or even eliminates the ability to activate phosphodiesterase; do you still hold to that?

DR. CORMIER: The aggregate is less active, but remember that work was done with peanut seed calmodulin. We don't see, in the case of mung bean calmodulin, the serious aggregation phenomena we saw with peanut seeds for whatever reason.

DR. ROUX: Do you think the aggregation phenomenon is related to having a free sulfhydryl there?

DR. CORMIER: No, because you can reduce it.

DR. ROUX: And it still aggregates.

DR. CORMIER: It still -- right.

DR. DELA FUENTE: Regarding your hypothesis of the control of calmodulin and oxygen evolution, you are saying that it is the increase in NADP. Actually you can test so there is an electron pathway going through NADP. Why can't you just provide an oxidized compound that would upset that.

DR. CORMIER: Yes, that is a good experiment, sure. That is an experiment that should be done. You are right.

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CORMIER FIGURES

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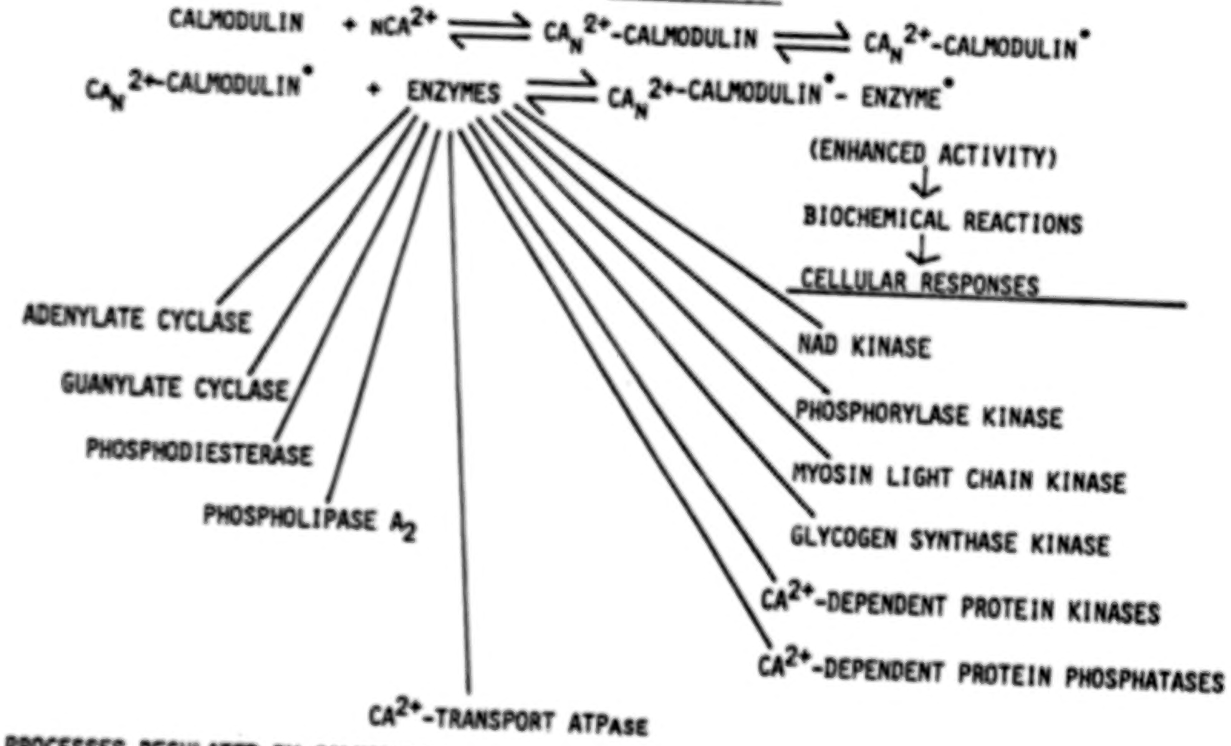
FIGURE ONE


CALMODULIN + NCA^{2+} $\rightleftharpoons$ CA$_N^{2+}$-CALMODULIN $\rightleftharpoons$ CA$_N^{2+}$-CALMODULIN*
CA$_N^{2+}$-CALMODULIN* + ENZYMES $\rightleftharpoons$ CA$_N^{2+}$-CALMODULIN* - ENZYME*
(ENHANCED ACTIVITY)
BIOCHEMICAL REACTIONS
CELLULAR RESPONSES
ADENYLATE CYCLASE
GUANYLATE CYCLASE
PHOSPHODIESTERASE
PHOSPHOLIPASE A$_2$
CA^{2+}-TRANSPORT ATPASE
NAD KINASE
PHOSPHORYLASE KINASE
MYOSIN LIGHT CHAIN KINASE
GLYCOGEN SYNTHASE KINASE
CA^{2+}-DEPENDENT PROTEIN KINASES
CA^{2+}-DEPENDENT PROTEIN PHOSPHATASES

PROCESSES REGULATED BY CALMODULIN:

1. CELLULAR MOTILITY (ACTIN-MYOSIN INTERACTION; ACTIVATION OF DYNEIN ATPASES)
2. CYTOSKELETAL FUNCTION
3. GLYCOGEN METABOLISM
4. CYCLIC NUCLEOTIDE METABOLISM
5. CALCIUM METABOLISM
6. PHOTOSYNTHESIS

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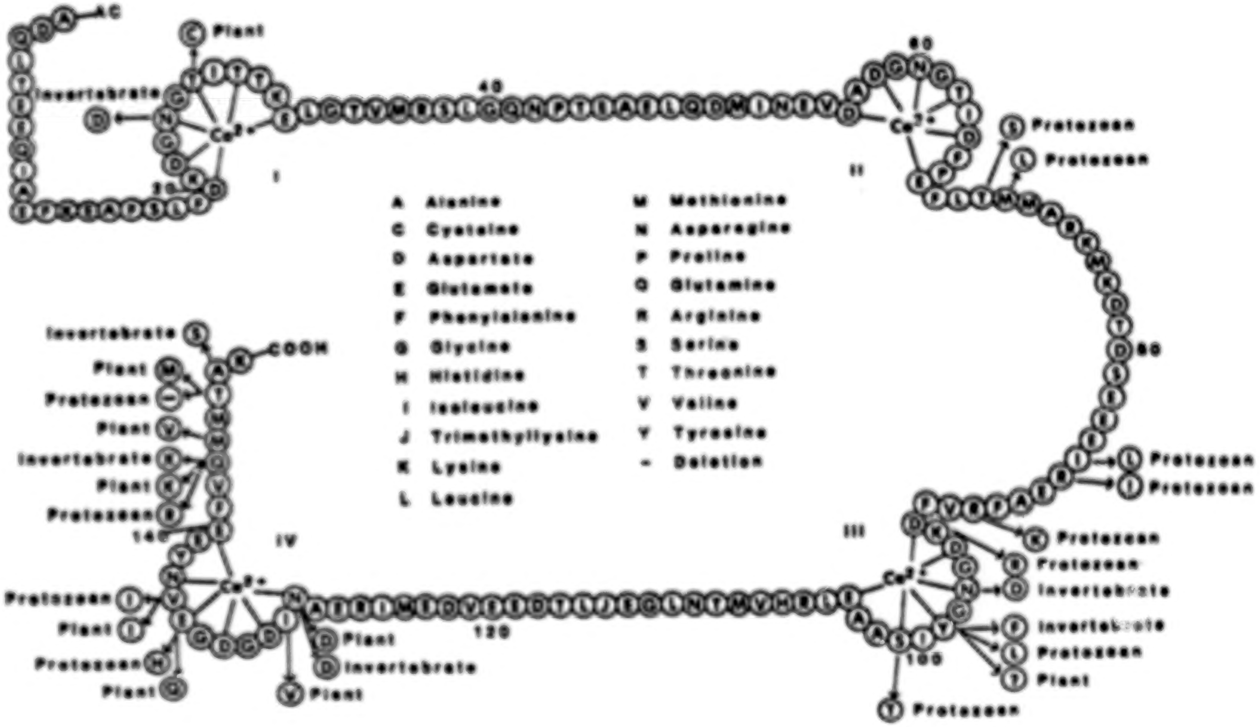
FIGURE 2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I
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Plant
Invertebrate
Protozoan
A Alanine
C Cysteine
D Aspartate
E Glutamate
F Phenylalanine
G Glycine
H Histidine
I Isoleucine
J Trimethyllysine
K Lysine
L Leucine
M Methionine
N Asparagine
P Proline
Q Glutamine
R Arginine
S Serine
T Threonine
V Valine
Y Tyrosine
- Deletion

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Cormier Figures

FIGURE 3

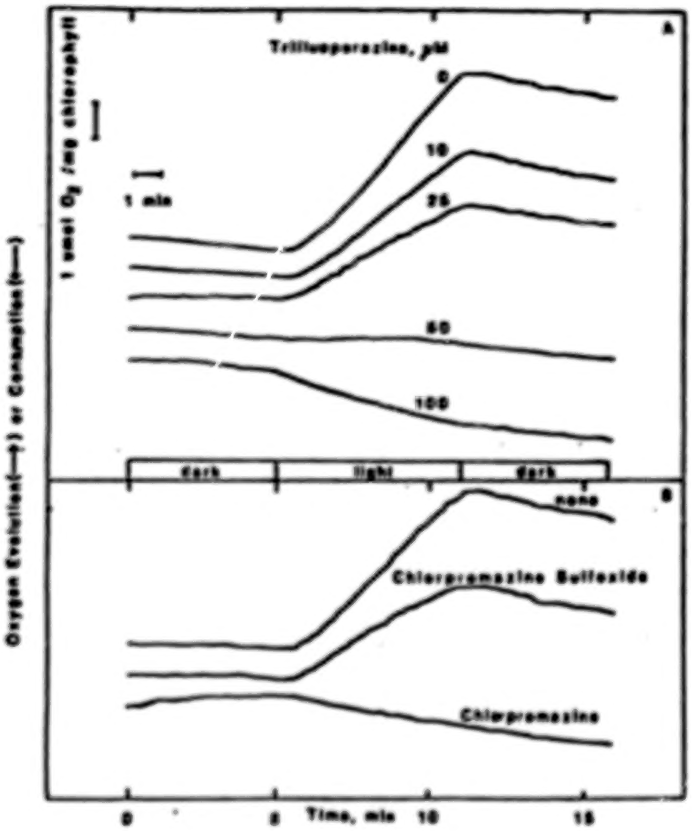
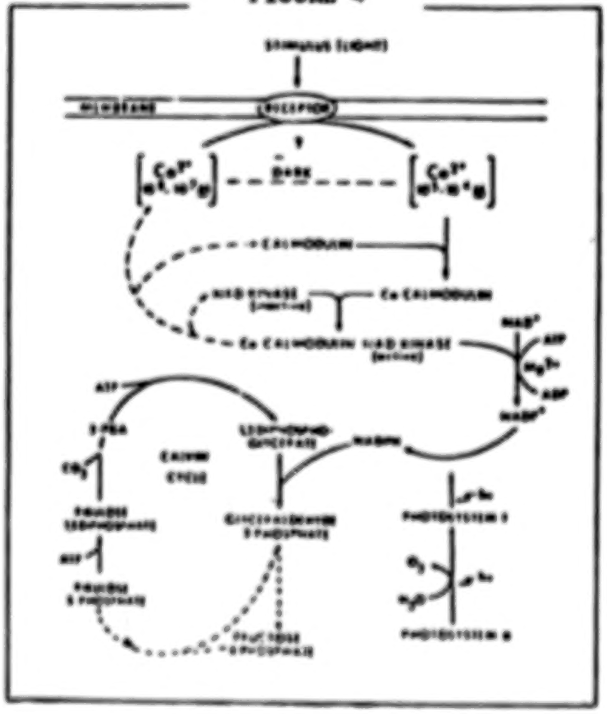


FIGURE 4


STIMULUS (LIGHT)
MEMBRANE
RECEPTOR
DARK
CALMODULIN
Ca CALMODULIN
NAD KINASE (inactive)
Ca CALMODULIN NAD KINASE (active)
NAD+
ATP
ADP
NADP+
NADPH
CALVIN CYCLE
PHOTOSYSTEM I
PHOTOSYSTEM II
O2
H2O

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DR. ROUX: Thank you very much. Our second speaker this afternoon will be Dr. Kenneth Robinson from the University of Connecticut Health Center and his topic will be local calcium entry and guidance of growth.

DR. ROBINSON: A lot of the work that I am going to talk about here today was done when I was a graduate student at Purdue toiling away in the subbasement. I managed to remain spectacularly ignorant of a lot of the work that was being done by people that have been mentioned here today, Tom Hodges and Leonard and Heven Sze, D. J. Morre, but the occasional breaks that I did get from that work in the subbasement was when Carl Leopold would invite me out to his luxurious country estate to play a little bit of tennis.

My interest, my intellectual interest, is in the question of how pattern arises; how does the pattern of the developing organism and of the adult develop out of the relatively unpatterned egg, and what are the signals and communication mechanisms between the different components, initially, of the single cell of the egg and later on of the embryo as a whole that allow a pattern to arise out of lack of pattern. This is what I think is clearly a case of a non-genetic mechanism, in the sense the genes simply do what they are told and make the proteins that they are told to do at the appropriate times and in the appropriate places. The question is how does a particular cell know that it is in the appropriate place or how does a particular region of a particular cell become different from the other one in the other region?

A classical organism that has been used by developmental biologists to study this phenomenon is that of the *Fucus* egg. *Fucus* is a seaweed, a brown alga. It grows on rocky shores. It is a common rockweed found on both coasts and in cold rocky waters everywhere around the world, I guess, but it has the interesting property from a developmental biologist's point of view that by the time the egg is released from the plant and is fertilized it doesn't seem to have any pattern built into it yet. It does not have any of its axes specified. That is unlike the case of animal eggs which in general by the time they are released from the ovaries and are in a fertilizable state already have some degree of pattern built into them, at least a head/tail axis specified and this occurs during oogenesis. The development of this pattern occurs in the ovaries where it is relatively inaccessible for study, but in the *Fucus* egg, this happens after the egg is fertilized out in seawater where it can be studied, and it can be controlled to some extent by the experimenter. So, people have been studying polarity in *Fucus*, for at least for 100 years, since the 1880's, and considering these questions.

The oogonium of a *Fucus* is just a capsule that contains eight eggs as they are released. By the way, it is an unusual plant cell in another regard. It has no cell wall at the time it is released and fertilized. That capsule dissolves in the seawater, giving a naked egg with a nucleus that is centrally located. The *Fucus* egg is very densely pigmented. Optically you cannot see anything inside it which limits what one can do with it. There are species where the male plants and female plants are separate. So, you can get isolated eggs and isolated sperm and control fertilization. You mix them together. The sperm swim around on the surface of the egg and fertilize it. It is all quite like any respectable animal. I mean you could show this, I think, to a lot of biologists, and if the optics weren't too good, they couldn't tell you whether they were looking at sea urchin or *Fucus*.

Now, here is the interesting part. Some hours after fertilization but before there has been any cell division these eggs develop an axis and this axis is manifested by a local bulging out, a local growth at one point and this represents the formation of the rhizoid of the plant, and after further development this rhizoidal cell is then cut off by a cell wall that is perpendicular to this axis, and the interesting thing is that any point on the surface of a given egg is capable of producing the rhizoid or any region I should say, not a point, and where it grows out is a matter determined by external vectors that are applied. For example, the usual orienting influence is unilateral light, and if you put the eggs in light from this direction, then that will cause the rhizoid to form out on the shaded side. There are, also, a variety of other gradients which can polarize the egg in the absence of light although in general light is the most effective polarizer of the egg and is probably the natural polarizer. You can see how it makes sense from the point of view of the developing embryo to have its root growing down toward the rocks. This is the part that holds it down to the rocks that it grows on.

I am going to refer to this later. If you simply put these things in a fluorescent microscope with FITC optics, the chloroplasts are brightly fluorescent. This is the cell taking its own picture. This red fluorescence fades out and is replaced by a kind of yellow-green fluorescence which is also, from the chloroplasts and if you fix these cells they are chock full of phenolic vesicles so the fixed egg, also, has a lot of autofluorescence at yet different frequencies. I mention all this because I am going to get to some of our difficulties with trying to do various kinds of optical studies on these cells, the fact that they are intensely autofluorescent at a variety of frequencies.

The real experimental part of my story starts with some work that was done by a superb plant physiologist named Elmer Lund who worked at the University of Texas (1), and I think this was published in 1923. What Elmer Lund did was put Fucus eggs in an electrical field simply by passing current through the medium, the seawater in which the eggs were growing, and he found that they showed a surprising tendency to form their rhizoids on the positive or anodal side of the chamber. This particular experiment led Lionel Jaffee a number of years later to consider the possibility that perhaps what the applied electrical field was doing was either modulating or mimicking an endogenous electrical field, that perhaps the eggs drove a current through themselves, and he devised what I consider to be a remarkably clever way of demonstrating that the cells did drive a current through themselves (2). He pulled them up into a capillary tube, so he would have several hundred eggs in a capillary tube, and then he oriented the entire population by shining light from one end. If the eggs are driving a current through themselves there will be a small voltage drop across each egg. If you line them up, those voltage drops will add up, like little batteries in series, and if you have several hundred you get therefore a several hundred-fold amplification of the signal. He was able to show using this method that, indeed, there is across a tube containing these eggs; if they are lined up, a potential difference develops, and he was able to infer from that what the size of these currents through the egg might be, and he found that there were currents that were consistent with what Lund found, in other words, with positive charge entering the growing tip, the growing rhizoidal tip. So, he suggested that perhaps part of the feedback mechanism that was involved in polarizing these cells was electrical and I mention the word feedback because what you are doing is going from an apolar system, and the cell then is taking what seems to me, at least, to be a fairly subtle signal, unilateral-light and converting that subtle asymmetry into, as far as the cell is concerned, a gross asymmetry. You know, you are going to get two different ends of the plant, if you will the root shoot axis resulting from that light signal, and the question is how does the cell amplify that signal. Jaffe suggested that it might be electrical.

Now, his tube method, elegant as it was, did not allow one to get very good spatial resolution. You could not really tell where around the egg current was going in and leaving, nor could you get good temporal resolution. You had to use populations of eggs that were somewhat asynchronous. So, ultimately that was a limited technique, and he and Richard Nuccitelli then developed the so-called "vibrating probe" for doing this (3). The vibrating probe is simply a glass micro-electrode filled with metal, just as a conductor, with a platinum ball plated on the end. This whole arrangement is attached to a piezoelectric element and is vibrated between two positions in the medium.

The point of this is that the platinum has a very high DC resistance, but it is capacitatively coupled to the medium so that at even a few hundred hertz AC, its impediance drops dramatically, and its noise which is proportional to the square root of its impediance, also, drops and allows you to get noise levels low enough to measure the kinds of voltages that these cellular currents might produce and, in seawater we are talking about perhaps 10 nanovolts of signal. So that is why one had to go to this extreme length and why conventional KCl filled electrodes wouldn't work. It was five years from the time it was first conceived until any measurements were done with it, and they were able to use it to successfully map the currents around the Fucus egg. I have always been grateful that they did this, and that they started with the Fucus egg because this must be growing in seawater so the signals are very small. The kind of voltage differences you get between two points depend on the conductance of the medium. So, for a given current the voltages that you get in seawater are about as bad as you can get because seawater is about as conductive as any medium that cells grow in.

So, in a sense, for many purposes and for my own purposes now working with amphibian embryos the thing was enormously overengineered, and a slob like me can come along and use this thing and get good measurements, since they designed it to do something so difficult, but using this they were able to actually map the electrical field around the developing egg, and these current lines that you see on the outside of the embryo here are actual measurements. The probe was put in, and they did two-dimensional measurements and constructed a map of the field around it. Now, the field lines on the inside of the cell, of course, were not measured. Those were simply inferred. The current lines have to be continuous, and you can apply some degree of potential theory to make some assumptions and draw them, but the field lines on the outside are measured, and what they found was that current enters the growing tip. Furthermore Nuccitelli has subsequently shown that this current begins even before the egg germinates which was not obvious from Jaffe's tube measurements, I think because the eggs are somewhat asynchronous and so on, and so the currents precede the actual formation of the rhizoid and indeed precede the fixation of the axis. Initially this axis, this rhizoid-thallus axis is labile. You can give the eggs a pulse of light, and then there is a period of time in which you can reverse the axis simply by giving it a reverse pulse of light, but then you reach a point before it actually grows out where you can no longer reverse the axis, and the currents begin even before the axis is completely fixed.

The question then is what ions are involved in these currents. These currents are so small and the nature of these vibrating probe measurements is such that there has never really been a clear-cut case where one could infer what ions are driving the current through a biological system simply by doing pharmacological experiments or by deleting ions from the medium and so on.

It has been a tricky business to get at that, and I began to look at this using a different method. I took nickel screens which actually were even finer than this diagram shows. They were about 20 microns thick while these eggs are about 100 microns, and one can take a slab of this screen, a circle about an inch in diameter and fill every hole out with an egg, for a total of about 25,000 eggs. Then I could polarize the entire population by shining light from below or from above and make it so that on one side of the screen I either had all rhizoid or all thallus. Furthermore I could do these experiments before there was any change in shape. I have shown it with a shape change here just for dramatic purposes, but I could do this experiment before the thing actually grew out, and therefore I would have a symmetrical circumstance. I would have a circle here on this side of the screen that was going to be thallus and a circle, an equal size circle here that was going to be rhizoid, and I would know which was which by knowing direction I would shine the light, and then I could put this in a chamber pass radioactive medium over it and measure, for example, the uptake, the rate of uptake of radioactive ions from the medium. Obviously I would not be talking to you if I hadn't done it with calcium, which of course I did, and I will get to the results in just a moment. This is just a face view of such a screen, each hole of which is filled with one of these eggs,and when I did the measurements with calcium, and I got these results, I will have to explain a bit how these results were presented.

First of all, I was measuring the flux of ions, and I went to substantial lengths to guarantee that I was really measuring a flux, that is the rate of entry of calcium, and while the eggs are difficult to work with in some ways, they are pretty big for a biological cell, and you can carefully wash them so you get rid of the extracellularly bound calcium which is a tremendous problem, and you don't change the calcium inside very much. So, if you give them, say, a 15-minute pulse of calcium, you can wash them for a long time, get rid of all the calcium that bound to the outside, but the calcium that went in during that 15-minute pulse. Very little of Ca will come out, and also, 15 minutes is short enough that you are still on the linear part of the uptake curve. So, you can do real pulse labeling experiments and are getting rates of entry, or rates of entry or rates of efflux. What each of these points in Figure 1 represents is the result of a pair of experiments measuring the influx at the rhizoid or the future rhizoid and, also, the influx at the thallus in a different batch and then comparing the ratio. These were equal areas being measured, area isn't involved.

By the way these eggs hadn't actually visibly germinated. They don't change, that is, start bulging out until about 20 hours after fertilization. The axis becomes fixed somewhere in this region, and for technical reasons 6 hours was about as early as I could do the experiment, but starting at 6 hours what I found was that there was a tremendous difference in the rate at which calcium went into the two ends of the egg (Figure 1). Five times as much calcium entered the rhizoid as entered the thallus, and what was a surprise to me at least it seemed that calcium came out the thallus at a greater rate than it came out the rhizoid (4). I did not expect that. You know, you could create a calcium gradient or a calcium current through an egg just if you had the influx localized. You would not necessarily have to have the efflux localized, but what these data indicate is that there is a genuine calcium current through the egg, a flux of calcium through the egg. Calcium is entering one end of the cell, the part that will become the rhizoid and is being pumped out the opposite end of the cell, and this led us to suggest a kind of calcium gradient hypothesis of growth. The idea was that somehow or other calcium is involved as a link between the light signal and the growth response, and one thing that the light signal does then is open up calcium channels or perhaps move calcium channels around, at any rate making this end more permeable to calcium than this end (the calcium pumps seem to be localized at the efflux), and we suggest that the result of this would be the creation of an intracellular calcium gradient. There has to be a gradient in calcium. If you are leaking it in one end and pumping it out the other, and it is moving through the egg, and the total calcium isn't changing inside the cell, which it doesn't, then there has got to be a gradient. The question is how big is that gradient. We think that it could be substantial. Calcium is certainly quite immobile in cytoplasm as shown by a lot of methods. So, we think the possibility for substantial calcium gradient in the cytoplasm of this egg exists. What this suggested was that one could polarize the eggs if you imposed a gradient of calcium on them.

Now, you might think, thinking of my screen method, okay why don't you just put low calcium on one side, high calcium on the other and see which way they grow? I tried that, and I got quite puzzling results. In fact, I got backwards results from what you would expect. I am only mentioning this, by the way, as a kind of cautionary note, and rather than give up my idea I looked for an alternate explanation. What I did was measure the permeability of these eggs to calcium as a function of extracellular calcium, just by giving them pulses of calcium and measuring the change in uptake. What I found was that the cells are spectacularly sensitive. Their permeability to calcium is spectacularly sensitive to the calcium concentration and they are inversely related (5).

In other words, the calcium is reduced from its normal level of 10 millimolar which is what it is in seawater down toward 1 millimolar. The calcium permeability goes up. If you go from 10 millimolar to 2 millimolar, the calcium permeability goes up 50-fold (Figure 2). What that means is that you have reduced the driving force, the inward driving force on calcium five times, but you are still getting far more calcium in. You are getting, in fact, 10 times the rate of entry of calcium because the calcium permeability has gone up so much, and so reducing extracellular calcium and modulating extracellular calcium may not have the expected effect on calcium permeability.

I don't know whether this is true in any other cells or not, although I was intrigued by a recent paper where somebody was looking at hypertensive people and found to everyone's surprise that the free calcium, the ionized level of calcium in the serum of people with hypertension is lower than it is in the serum of people who are normal, non-hypertensive. You think of hypertension as affecting the smooth muscles of the blood vessels, and you would think that these people might have higher calcium in their muscle cells than the normals; yet, it was found that they have lower serum levels of calcium, and it put me in mind of this older data of mine which I published in a long forgotten plant paper.

As an alternate way of trying to impose a calcium gradient on the cell, I began to use the calcium ionophore A23187 and this is some work that I did with a student Roger Cone. What we did was take glass fibers and coat the outside of these glass fibers with the ionophore. That was done simply by dissolving the ionophore in a little bit of ethanol, adding a drop of ethanol to the fiber as it lay on a glass slide. It ran along the fiber. The ethanol dries and you are left with a rod coated with ionophore, and then you stick this down in the bottom of a dish, fill it with seawater and seed it with eggs and let the eggs fall down. The eggs near the fiber will experience a gradient of the ionophore. The ionophore dissolves very slowly into the seawater so that the fiber acts essentially as an infinite source, and you have got them in a large volume of water which acts as an infinite sink. So, the thing never comes to any kind of equilibrium during the course of the experiment which is about 10 or 12 hours, and I have shown that at the end of that time you have plenty of ionophore left on the rod. The ionophore is fluorescent, so you can see it.

An egg in the neighborhood of the rod will experience a gradient of calcium ionophore. One side of it will see a high concentration of ionophore compared to the other side, and I don't know exactly the steepness of the gradients, but some theoretical modeling suggests that an egg sitting within one egg diameter of the rod might see a four or five-fold concentration gradient, at least a twofold concentration gradient of calcium ionophore, and what we found was that this was quite a strong polarizer of the eggs (6).

If you just look at the eggs near the fiber, you will see (Figure 3) that most but certainly not all form their rhizoids toward the rod, and of course, the way we really did the experiment was we looked at hundreds of eggs, measured the angle of this axis compared to the normal and then calculated an average cosine and so on, and you can show very clearly that the calcium ionophore is quite a strong polarizer of the egg. As a control, for example, valinomycin the potassium ionophore has no such polarizing influence on the eggs.

We wanted to know is there really a gradient of calcium within the eggs as we say and if so, how much is it? Some years ago we did not know how to do this. I mean this was after aequorin was invented and everything, but nevertheless as I have pointed out there are some real technical difficulties with these eggs, and I think the egg is too small to inject a single egg with aequorin, image it with an image intensifier, and be able to measure the light from one end of the egg as opposed to the other. I just don't think there is enough light. You cannot get enough light to do that. Quin 2, had not yet been invented, it was still a gleam in somebody's eye, I suppose. So there really wasn't a way of doing it, but as a poor substitute we wanted to look at the distribution of total calcium. This is work I did with Larinda Jaffe and John Gilkey. What was done was the eggs were exposed to radioactive calcium for some hours, as they were growing. They were then frozen by plunging them into liquid nitrogen, sectioned with a cryostatic sectioner and then median sections were put on autoradiographic film, all being done at low temperature, so that everything was kept frozen the whole time. You let the autoradiograms develop and you look at them, and the lower three autoradiograms here are calcium autoradiograms, and I think you can clearly see that the grain density at the rhizoid is higher than it is at the thallus, and we confirm this both by grain counting and by densitometer scanning and the difference between here and here is something like three-fold. So, there seems to be perhaps three times as much total calcium in the general region of the rhizoid as the thallus.

Now, someone mentioned earlier, maybe Stan, that the resolution of calcium on radiography isn't very good. You get some idea by seeing the grains out here where you can see the boundary of the cell but not very distinctly. So, we don't have great resolution with this, really, but good enough, I think, across a -- this is 100 microns, to see a difference between here and here. These are chloride controls. We wanted to make sure that this wasn't just some non-specific effect. So, we did do some chloride autoradiography, and it may appear that there is a little bit of a reverse gradient here but actually when we examined many eggs and did scans and averaging, it turns out that chloride was quite uniformly distributed throughout the cell. I have plans for and a whole different attack of how to get at this looking at ionized calcium, and maybe if I -- the next time I speak to some of our group I will be able to give those numbers, but we are working on that seriously now.

Just one last thing. We have been interested over the years in the question of how the cytoskeleton might be involved in this. It has been known for a long time that Fucus eggs will not polarize in the presence of cytochalasin although for example, colchicine has no effect, but they will not polarize with cytochalasin. It has been very hard to get at this, because these eggs are a terrible object for fixation for electron microscopy. They are chock full of phenolics and as soon as you fix the egg the phenolics start leaking out of vesicles. They disrupt everything. They partially fix things, and what you get is a real mess, and a number of people, some very good people, I think, have given up in disgust on this egg as something to look at. Susan Brawley who is a postdoc now in my lab, has demonstrated unequivocally that there is actin or at least a protein that comigrates with muscle actin in fucus eggs. But to actually try to look for it and see actin microfilaments in the cytoplasm has been very, very difficult. The recent development of NDB phallacidin which is a fluorescent derivative of phallodin which is a fungal metabolite which binds to F actin, filamentous actin, has simplified this.

The NBD phallacidin is fluorescent, and you can use it with classical cells, tissue culture cells and show that it really lights up the actin microfilaments very nicely. The problem with this cell, doing something fluorescent with it, of course, as I mentioned earlier is the cell is autofluorescent. So, in order to see something you have to use very narrow pass filters. You have to throw away an awful lot of your light. If you examine a bright field micrograph of a Fucus egg which has been treated with NDB phallacidin, I think you can see, even though there is this general level of background fluorescence, a bright staining at the tip.

The control was treated with the NBD phallacidin, plus a 50-fold excess of phallodin which is not fluorescent, and this gives just the background staining. You get this same staining if you don't use any fluorescent on it at all, and that is the best we have been able to do, but there is a suggestion here that this binds only to F actin not to the free actin molecules but only to the filamentous actin and that there is an accumulation of F actin in the growing tip. Such things have been suggested for other tip growing plant cells.

I am going to quickly run through something to try to convince you that this isn't just a part of the bizarre case of a Fucus egg, that some of these ideas have more general applicability. I am working now with the Xenopus embryo. Xenopus is a frog that comes from South Africa and it is quite a popular experimental object because they are hardy, and accessible. The eggs are very big, unlike Fucus eggs which are 100 microns. These are 1500 microns, and they develop rapidly. A frog is a classical embryological system as well.

By stage 16 the neural tube has started to form. The neural tube is formed by a wrapping around of the neural plate to form a tube, and this will become the spawning of the animal. Here is a slightly later stage of this. This is about stage 19 or so. With these stages that we work, we simply cut out the dorsal one-third and we can extract from that the neural tube which is just formed. We can actually just pull it out rather cleanly, put it in divalent ion-free medium, get these cells to disaggregate. After they are disaggregated put them in culture where they settle down and a substantial fraction will then grow neurites.

Now, the question of whether nerves respond to electrical fields is an old and a controversial one. It is controversial because there were many early reports that they did. Paul Weiss tried it, and he had a powerful influence on American neurobiology, and he said they don't at all, flatly said that they didn't and that settled the issue for 30 or 40 years literally. There have been conflicting reports for a long time. I think the reason is that the people who did these things and tried to grow them in electrical field were working not with single neurites but with intact ganglia and out of the ganglion you get, instead of getting one or two neurites like that, you get a whole halo of hundreds of neurites and there are fibroblasts in there, and analyzing what is going on in a ganglion is very difficult.

This work was done by Colin McCaig who was a postdoc in my lab. We wanted to see if these cells would respond to an electrical field. So, these cells were grown in an electrical field, the negative electrode over here; the positive electrode is over here, and what we found, and I chose this slide because it shows three different responses that we saw. If a nerve starts out to grow perpendicular to the direction of the electrical field it will curve toward the cathode (Figure 4). If it starts growing parallel, if the neurites start out parallel to the field, the one growing toward the positive electrode is very much shorter and in fact often gets retracted into the cell and the one growing the other way is stimulated and if the neurites branch they branch toward the cathode, as well. By the way, these cells grow by tip growth. The mechanism by which they extend and elongate is quite similar to a tip-growing plant cell which Fucus, as I have shown you earlier is. Furthermore, we found that this occurs at extremely small electrical fields. By analyzing the direction of growth here compared to the initial direction of growth for hundreds and hundreds of cells and then varying the field strength we were able to evaluate what the threshold for this effect was, and these cells can respond with growing nerve, can respond to electrical fields, well, a field such that the voltage drop across the growing tip is something like 300 microvolts. For these cells that is a field of about 7 millivolts per millimeter.

Down near the threshold. This is the sort of thing you see. This side actually grows faster than this side. There is a stimulation, and it will, also, curve. By the way, these results have recently been confirmed really quite completely by Mu Ming-Pu and Patell in California at Irvine.

Surprisingly we, also, found that muscle cells from these same embryos respond to applied electrical fields by lining up perpendicularly to the applied electrical field. They start out spherical and then form elongated muscle cells which become striated, can be innervated and in fact, they are quite like they are in the intact animal. In Xenopus the muscles don't fuse with each other, these embryonic muscle cells that drive the tail of the tadpole. Then to my knowledge no one had ever reported a response like this to an applied electrical field. So, the result is that you can get the nerves in culture to grow perpendicular to the axis of the muscle cells. You can see the striations of these muscle cells which mimics, actually what happens in the intact tadpole.

So, this is the growth cone which I think is the part of the nerve that is actually responding. This is the growing part of the nerve, and I think it is this growth cone that is responding to the applied electrical field.

I was interested though in whether local calcium entry played a role in the growth of the nerve. So, since the geometry is somewhat different, what I used rather than a rod coated with ionophore, was a sealed microelectrode and simply coated the tip of the microelectrode, the outside of the sealed microelectrode with calcium ionophore. Here is a neurite growing past the cell here and this is the growth cone which is the active region and we bring up the tip initially not really closely enough and you see it start to respond but then as often happens it retracts back a bit, but then, and these are each about 10 minutes apart, it starts growing again and it makes quite a sharp turn toward the source of the ionophore, and this is quite repeatable, and again, at least valinomycin has no effect on this response. So I suggest the possibility at least that the growth directional response of nerves involves local calcium entry and perhaps this is a common feature of tip growing cells. You might ask how do these cells interact with an electrical field? Well, one possibility that we have no evidence for whatsoever but nevertheless I like is that we have shown that it is possible to move proteins in the plane of cells by externally applied electrical fields. This is the so-called phenomenon of lateral electrophoresis. We did it, Mu Ming Pu and I did this originally with the Con A receptors of muscle cells, and he has subsequently done it with the ACH receptor of muscle cells, shown that you can move them from one side of the cell to the other just by applying an electrical field. They are, in a sense being electrophoresed in the plane of the membrane, and one possibility is that with, the external electrical field that I apply we are actually moving calcium channels from one side of the cell to the other. In summary, I think that one mechanism whereby cells polarize and respond polarly to external stimuli is by way of local calcium entry, that I think I have shown that at least in one case that local calcium entry does occur and secondly in a second case that if you force local calcium entry you get a response.

Now, I will leave you each to your own private musings, and I am sure you will each have different ideas about then how any kind of resultant calcium gradient in the cell might then further act to produce the growth response, but I am sure there is no shortage of possibilities.

Thank you.

DR. ROUX: Questions?

DR. BIKLE: I am a little confused about the calcium storing and polarity, the electrical field. In one case we had the rhizoid going toward the positive pole, and in this case we have the neurite going to the negative pole.

DR. ROBINSON: Right.

DR. BIKLE: Both going toward the 23187. So how do you put that together?

DR. ROBINSON: Yes. That is true, and I have no obvious explanation except to say that these are different cells. We don't really know how the electrical field is interacting with the cells. My lateral electrophoresis is only one possibility, but one could imagine if you, just for the sake of concreteness, consider the possibility that channels are being electrophoresed. You could imagine that they would go one way in one cell type and another way in another cell type. Seawater organisms versus fresh water. Evolutionarily they are a zillion years apart and everything, but I don't have an obvious answer for that. Really I don't, is the answer, and you are perfectly right to point out that that is a paradox.

DR. HEPLER: Since the ionophore is fluorescent, have you looked at the growing neurite with the fluorescence microscope?

The reason I am asking this, you might, to explain the bending there, you might expect to see a patchy fluorescence along the growth cone side on the direction towards the --

DR. ROBINSON: You might. I don't know whether there is enough of it that dissolves off into the medium and gets into the membrane that you could ever see it. I kind of doubt it. I mean you can see the source because you are putting a lot of it on it, but the damn stuff is very insoluble in water, and how much of it gets into the membrane, whether -- I have not looked.

DR. HEPLER: It might pile up in the membrane. It might collect there, you know.

DR. ROBINSON: I suppose it is possible.

DR. MALACINSKI: With regard to the rhizoid formation of Fucus, I like the way you expressed yourself that it appears that calcium is a link between the signal light and the response growth and you are implying that the first effect is really a light effect. Would you care to speculate on how light generates an asymmetry in the ion pumps and the ion channels if that is the first response?

How does that come about?

DR. ROBINSON: I have dreamed of schemes by which it happens, but we don't even know what the photoreceptor is. But, you know, you can imagine a direct effect. You can imagine, for example, obviously calcium is intimately involved in vertebrate photoreception and release of calcium is very close, at least, to a primary step in the reception of the light. Maybe a similar sort of thing happens here. What I do imagine is that even at that earliest step there has to be some kind of a feedback mechanism. Suppose the chromophore that is receiving the light in the Fucus egg releases a calcium molecule or doesn't release a calcium molecule because they grow out on the dark side and that somehow or other shuts off the calcium -- the released calcium shuts down the calcium permeability or something like that which sets up a small asymmetry. Then there has to be a mechanism to amplify that, and I don't know what that might be.

DR. MALACINSKI: But presumably you can stimulate a rhizoid outgrowth without light. There are other ways.

DR. ROBINSON: Oh, yes, you can do it with apparently no gradient at all and presumably what they are polarizing on them is the point of sperm entry, and at least in other species there is evidence for that and so --

DR. MALACINSKI: Whatever mechanism redistributes the initial asymmetry, I don't think you have to, a priori, imply that it is light driven because it can be mimicked in other ways and so the search for the mechanism shouldn't focus so much on the chromophore.

DR. ROBINSON: They are powerfully sensitive to light, and the tiniest amount of light easily overcomes all these other asymmetries. So, what I suspect is that the other asymmetries somehow or other weakly mimic whatever light does. Sperm entry in lots of eggs, in animal eggs, at least, induces a release of calcium. Perhaps by bringing in a bit of calcium of its own, perhaps by making the membrane leaky to calcium; it is not quite clear, but there is no question that following the fusion of the sperm to the egg there is a tremendous release of calcium inside the cell. I have some evidence from studying an entirely different phenomenon that the *Fucus* egg is at least, its electrophysiology is similar to animal eggs as far as fertilization is concerned. So, that something like that could happen isn't impossible. I would suspect that the primary mechanism is light and that these other things are kind of weak imitators of whatever light does.

DR. KRIKORIAN: Having initiated the whole business, as with fertilization, if you were to put this on a clinostat, since you have got the means whereby you have got the nice mesh; can you neutralize gravitational effects, as it were? Can you keep it in the non-rhizoid-producing form for extended periods of time or is that not practical to carry out?

DR. ROBINSON: You mean use a clinostat to zero out light gradients or --

DR. KRIKORIAN: No, to zero out gravitational cues because gravity can be one of the cues, also.

DR. ROBINSON: I don't think anyone has ever tried to grow fucus in a zero gravity situation. One of the weak polarizers of these eggs, as Peter Hepler reminded me this morning, is gravity itself. You can centrifuge the eggs and that will determine the axis of the rhizoid, but again that is one of the weak ones. That is easily overcome by light, and you can centrifuge them so hard that you stratify all the contents at one end, but still you can come in with light, and you have got this egg that is stratified inside, and you can make it grow out from any point you want with a light pulse.

DR. THOMPSON: Is your EM fixation of the cell at least good enough so that you can say that what seemed to be that accumulation in the rhizoid of calcium 45 was not just due to something like a movement of mitochondria or something that --

DR. ROBINSON: It isn't really, and it might be due to an accumulation of mitochondria in fact, and I cannot rule that possibility out, and there are organelle redistributions.

DR. ROUX: Thank you, Ken.

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ROBINSON FIGURES

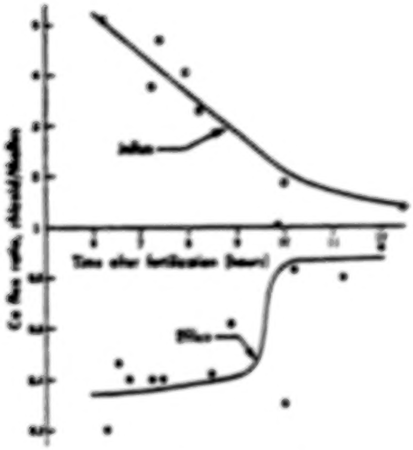

Figure One. Calcium influx and efflux ratios in developing *Pelvetia* eggs. Each point is the result of two simultaneous flux measurements on half-eggs made by using a pair of screens as shown in Fig. 1b.

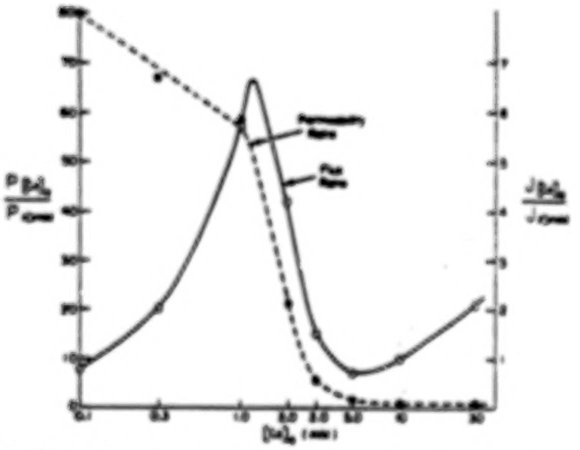

Figure 2. Calcium flux and permeability ratios as a function of $[\mathrm{Ca}^{2+}]_o$. The solid curve shows the Ca^{2+} flux divided by the flux at 10 mM $[\mathrm{Ca}^{2+}]_o$. The marked points (open circles) were calculated from the 5-min point of the curves in Figure 1, except for the point at 5 mM which came from a separate experiment. For an explanation of the interpolation between the points, see text. The permeability ratios (solid circles) were calculated by dividing the flux ratios by the corresponding concentration ratios

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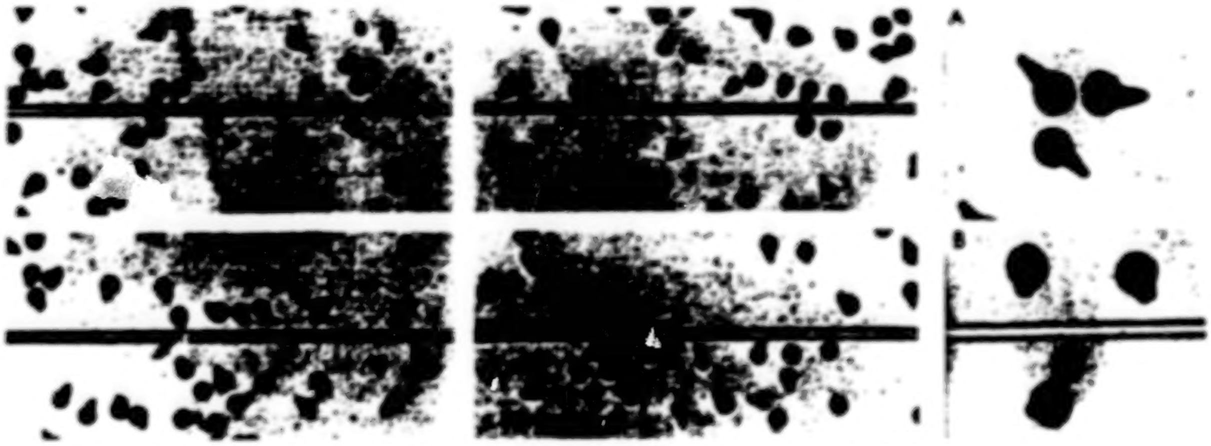
Robinson Figures

Figure 3



(left). Eggs adjacent to sections of an A23187-coated glass fiber. The eggs were placed near the fiber 1½ hours after fertilization; these photographs were taken about 24 hours later. The average short diameter of the embryos is 90 µm. Some 78 percent of the embryos within one egg diameter of the fiber have formed their rhizoids on the sides near the fibers; 60 percent of the remaining embryos have done the same.
(right). Differences in rhizoid morphology between (A) eggs more than 1 mm from the glass fiber and (B) eggs adjacent to the fiber. The photographs were taken at the same time and show embryos from the same dish.

Figure 4

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DR. ROUX: We are at a kind of a transition point in the workshop now. Before moving into talks which deal exclusively with animal systems we want to have four relatively short talks which broaden the information available that we have heard so far about how gravity affects the way plants and animals grow and how it affects their physiology, and the first of these talks will be given by, Abe Krikorian, and the title of his talk will be Developing Higher Plant Systems in Space.

DR. KRIKORIAN: My lab has been concerned for some time in developing potential test systems that might be exposed to hypogravity or microgravity environmental conditions. We are concerned with a number of problems involving morphogenesis, but unlike the elegant Fucus system that Dr. Robinson described, we want to work with higher plants whose cells are much smaller. We are dealing with cells on the order of 5 to 10 microns in diameter usually. We felt at the outset of our studies some time ago that one should in a typical space environment experiment deal with a rather simple system and ask very simple and fairly direct questions.

The first one that we addressed was: Can a higher organism, such as a flowering plant, achieve the degree of complexity that it ordinarily would, for instance, in the embryo sac in the developing ovule?

As much as one might like to work with direct micromanipulation, this is not an easy task to undertake, and insofar as I know, no one has ever been able to take the fertilized egg out of the embryo sac of a flowering plant and manipulate this to complete growth into an embryo or plantlet.

On the other hand, we have had some opportunities to develop systems concerned with the cloning plants (1), as it were, from single cells. I am going to address myself to this and give you a broad thumbnail sketch this afternoon on some of the kinds of tests that we have been able to carry out in real space environments. I hope some of these points I raise will be relevant to the overall theme of space biology experimentation.

You will recall that higher plant, and even lower organisms usually show some considerable polarization of their cellular components. In most cases this highly polarized format precedes the subsequent setting into motion of a number of events. We decided some time ago that it would be useful to develop systems at different levels of initial organization with the hope that we could pinpoint where any potential problems in the space environment might actually arise. The systems that we sought to develop and are continuing to do, involve protoplasts, that is to say wall-less cells of higher plants, free cells, organized units with minimal degrees of development, of somatic embryos with discrete growing points of shoot apex and root apex and, finally seeds with their contained pre-formed embryos (2).

The systems that we use involve taking virtually any somatic cell from any higher plant and under the right circumstances we induce these to express competence or their totipotency. Free, single cells rigorously isolated from cells grown in suspension can be plated out in a agar environment much like a microorganism, and these in turn will embark upon their continued growth and development as somatic embryos.

Now, in the case of the carrot we have several very real advantages, especially if we consider kinds of conditions that obtain on spacecraft.

First of all, not having access to light in the early stages of flight experimentation, we are able to take individual cells, expose them in complete darkness and end up with a substantial amount of morphogenesis.

While the shoot primordia form, their continued development is, not surprisingly, arrested, but nevertheless all of the apical cells and so, are all in the appropriate location.

Secondly, a major achievement is that in certain cell lines we can pull out clones and populations that will continue their development in absolute darkness. Coupled with this we can subject the cells prior to this event to cold. By keeping the temperature at around 3 to 4 degrees or even slightly lower, we can prevent any subsequent development from occurring (3). This was a very useful achievement in the sense that we want to test whether or not cells can continue to grow and multiply from one initial free cell. You subject it to cold, stop it in its track, as it were, keep it in suspended animation (Figure 1). Then, if you allow a permissive temperature (hopefully on the spacecraft) to be achieved, then the subsequent development can occur.

In the case of the carrot, we have been able to work this out. We prepare our cells, distribute them in small 50 millimeter diameter petri dishes. These can be stacked up in very small containers (yet another advantage under spaceflight conditions) where we have them in a canister the size of a tomato soup can, as it were. Nine dishes containing cells at different degrees of development (Figure 2). In one instance we used free cells. In other instances we used cells that had gone through a number of stages of embryonic development and others that had gone through still later stages of embryonic development.

You can see some of these polar divisions that have occurred in the embryos of the carrot. Even in cases where this polarization is not exaggerated as in this instance, one of the monocotyledon sytems we have worked with, one does, in fact, see subsequent polarity. So, no matter what happens morphogenetically, there is going to be some kind of polarity whether it is at the free cell level or even at the later developed stages of embryogenesis.

This business of exposing cells to the hypo G environment was worked out for cosmos 782 and cosmos 1129, and I guess it was initially something like 19-1/2 days we were able to get things back. That in itself was an achievement, I felt. The development had progressed to the equivalent state that it would have been achieved under conditions of 1 G. This experiment was further made more credible by the presence on board of the 1 G centrifuge. This is a very useful thing, and NASA has been trying to push this for a long time, for in any kind of hypo G or micro G environment, it would be very useful indeed to have a centrifuge on board.

We were lucky on the Soviet Cosmos 782 spacecraft in having access to the centrifuge, and this gave us very good controls (Figure 3).

When we compared control and flight materials we were unable insofar as we were able to carry out the measurements to find differences. There were no detectable differences, and we were quite positive from our ground controls that no cell divisions had been achieved prior to the lift off of the spacecraft.

Everyone has preconceived notions, and ours was that under conditions of 1 G where all evolution has occurred, it seems most peculiar that cells would behave and undergo morphogenesis in near hypo-gravity as readily as they seemed to do (Figure 5). So, I cooked up this idea. We could not eliminate the fact that there may have been something of a bit of a memory. A memory of the 1 G stimulus that these cells must have been exposed to in the 1 G environment on earth. There was some frog egg experimentation

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that had been done on some of the early American biosatellites that also pointed out that everything seemed to be okay. Even here, one couldn't eliminate the very real possibility that the cues whatever they might have been, and in this case gravity might be one of them, were not eliminated.

DR. ROBINSON: Don't you have the problem though of very large G forces?

DR. KRIKORIAN: That was another problem as well, and of course, in the Russian Cosmos satellite the G forces are very substantial in lift off and in recovery, and this is coupled, also, with very high so-called "spikes" of temperature, especially on recovery where you go up to things like 37 degrees for prolonged periods (2, 3). But by running the proper sort of temperature controls and going through what I call the "shake, rattle and roll," that is to say the vibrational simulation on earth, one can show that this did not, in fact, have any major impact on the stuff.

The other thing that I was concerned with at that time with getting such nice neat results was that the cells as we distribute them in this nice agar medium are highly insulated. They are almost like the buoyant egg cell in the embryo sac which is loaded with fluids. So, it is kind of floating and therefore not really as exposed to all the hazards, if one might term it that way of the space environment.

A good opportunity came up in Cosmos 1129 to repeat the Cosmos 782 experiment and extend it. We were able, again in a nutshell, to repeat that experiment and to extend it. We used cells and embryos that were quite advanced in their development. In both cases their continued development was achieved and to this extent, I believe that the results of 782 were completely repeated (6, 7).

The growing points on the plantlets, the inability to subsequently undergo continued growth and to flower turned out to be quite good. Their continued growth did not seem to be slowed down, and the normalcy of the growing point turned out to be pretty predictable. Some of the kinds of things that the Soviet investigators had pointed out, namely, rounding up of the cells in growing apices, were not encountered, perhaps due to the fact that in the very early stages again we are completely embedded in an agar environment.

The simulated events of embryogeny in cultured carrot will be discussed next. The free cells that one could expose to test conditions are not, unfortunately, covered by a very heavy pellicle as in the case of the _Fucus_. They are not as tough as _Fucus_ by any means, but they are still reasonable tough. We obtain these by filtration, as I said, select clones and filter out embryos at later stages; the early stages would be free cells as shown. Very large quantities are achievable. So, in a very small, limited space you have a fair amount of stuff for experimentation.

The temperature profile that I mentioned a while ago illustrates the added advantage, which is a very real consideration, is that you have to keep things status quo until the experiment time is reached. We were able to keep carrot cells viable and responsive for well over 60 days.

This was very good because inevitably the so-called "slips," to use NASA jargon, are going to occur. That is to say, unforeseen delays in the loading of the spacecraft at that critical moment, the very last moment possible, before they shut the door, as it were. That is a problem.

Dr. Alan Brown has handled this by his so-called "peacock," the plant carry-on container where an astronaut actually takes this and totes it on like a little suitcase.

So, in terms of biological experimentation one runs into these very practical considerations. That very elegant experiment isn't going to work if you cannot work within the confines, or at least if the spacecraft won't permit that type of manipulation.

The advantage of a very simple test such as ours was obvious in the case of the joint US-USSR flights. We used a very small, very simple, quite inexpensive piece of hardware. It has the added beauty of a temperature recorder which gives you a temperature profile, so that temperature can be recorded, one can rigorously establish that things could not have occurred without your knowing it, and since it is right in the package you don't have to worry about what the temperature restraints are on other parts of the spacecraft. That is another problem because in this kind of developing system temperature can be very, very critical.

I will speed right through the data from 782. We were able to go through 18 well-defined, albeit arbitrarily defined embryonic stages, and we did rigorous comparisons of these at the light and electron microscope level and didn't see any differences. They will flower. They will fruit and so on, and if you compare them, everything is worked out pretty well (6, 7). We include this slide to show that the cell did not have this rounded up appearance. There is something that must be addressed a little bit later on.

The 1129 repeat, a little bit of an indication on the insults that one sees. Don't get the idea that spacecraft environments are zero G. NASA made its mind up on that some time ago. You are never going to achieve a strictly zero G environment. At best it is going to be micro G until space platforms go up with big long tethers and umbilicals so that things are literally "out there". So, one cannot critically address the question of whether cells can undergo their first asymmetric division "normally," as if they would under 1 G conditions in a spacecraft as they currently exist. That certainly includes the shuttle which has 10^{-4}G space enviromental conditions. We can actually map the location of cell units that are put on a gas. We can get a very substantial degree of development in just a short period of time like 18, 19, 21 days.

Since we spent less time on things that I might otherwise have gone into a little bit more detail on, let me just point out that under the conditions tested, higher plants can achieve a substantial degree of organization under hypo G. But this hasn't been done for more than one part of a life cycle. One has to do this repetitively, in my view, before one can satisfactorily eliminate the possibility that there isn't this 1 G memory or the cue isn't there, or that the G cue which was initially active may still be there but inactive. I am sure you have a lot of ideas on what this kind of "memory" could entail.

The "bottom line" is that NASA - as much as it would have perhaps wanted to have a good clear-cut answer, cannot at this point in time say that one can achieve a full life cycle of a higher plant under hypo G conditions. There is no evidence that this can be achieved. This includes what the Soviets have attempted time and time again. They have been much more sympathetic to plant biology in terms of their flight program, putting lots of plant experiments on, bits and pieces but in no case has a plant life cycle been convincingly shown to have been achieved from "seed to seed". So, we don't know whether this kind of thing can occur.

Alan Brown, and at the same time Joe Cowles, have had experiments recently on the first two shuttles. These were not strict experiments. They were called "flight tests." In both cases we were fortunate enough through Dr. Halstead's intervention and the willingness on the part of Joe Cowles and his collaborators and Alan Brown and Dave Chapman to have material made available to us. The concept here was that it would be a nice if material which was being grown for whatever reason on a space shot could be examined from the perspective of another investigator. This would be very useful as long as the other, guest "investigator" or "piggyback person," as it were, did not interfere with the primary purpose of the experiment or test.

Allan Brown was testing the amounts of water or moisture content of the soil environment of sunflower seeds in his peacock hardware which is the prelude to the real Heflex experiment which will concerned with nutation. We won't go into detail on that, but we have had a longstanding interest in sunflower (Helianthus) cytology, and we said, "Look, do you think it would be possible to have access to some roots?" It worked out. We looked at the roots. Of five plants, three of these roots showed consistent aneuploidy, a fact which is a very, very unusual thing in sunflowers. No one has ever described an aneuploid sunflower, much less one where the entire root population in division, in metaphase was aneuploid (Figure 4).

The homologue of chromosome 6 in flight-grown material, in three out of the five plants to which we had access, was missing. We were quite excited about this. First of all and in hindsight, it shows it took quite a lot of temerity to think we would be able to get anything out of this because the roots were growing in soil. As you know, you cannot do cytology on soil grown roots very easily. But we were able, with some luck, to release enough of the roots from the soil environment to at least show that in the first cell cycle that is embarked upon after recovery from the flight environment that this sort of event is screwed up, as it were, if you will pardon the French. Whether this is a reflection of the space environment or not, we cannot address in a moment. The fact is though that this is a clear reflection of the first cell cycle after recovery. That is how it was timed.

We came across bridges which were again signs of real difficulties. In addition we came across Fuelgen positive globs that had been thrown out of the cells. What this means we don't know, but again the Fuelgen positive aspect of it certainly indicates that it is nuclear material. And this type of thing has only been described, as far as I know, in rather superficial terms in the literature where in the thirties centrifuged cells were examined in terms of their cytology (Figure 5).

I will make life very easy in the next minute or two by finishing. But before I do that, I would like to point out that again, we have had a chance to look at oat cells from roots that were grown by Cowles et al on their experiment which involved lignin synthesis and a few other parameters; again, whether this reflects the space environment we cannot say because we did not do controls on board and the fixation was done afterwards. But if one fixes with cytostatic agents prior to the time the first cell division occurs on earth as at the recovery site we found some very substantial aberrations in those cells that were in division. That came across - tremendous fragmentation, breakdown of the cytological features, almost an erosion, as I call it, of the chromosomes, very much like what would happen in the case of certain mutagenic agents under x-ray irradiation (8). Now, I might, also, point out, and this seems to be fairly consistent, that the spread of the chromosomes in the flight grown material, (while one cannot get a terrifically wonderful spread anyway in material grown under flight conditions), was very contracted (Figure 6). There is a fair amount of superimposition. I will finally just say that in the mung bean,yet another plant that we examined, seemed to be perfectly normal (Figure 7). In all instances though, the total number of cells that were capable of embarking upon division after recovery were much lower than would have happened under laboratory conditions - about half as many as would have occurred.

So, I will leave it at this point by saying that certain things seem to be fairly clear-cut. Whether or not we can do multiple life cycles is still a wide open question, and whether or not the environment of space, be it in terms of the hardware or whether it is the insults of flight or recovery; in this case we can only address a very narrow aspect, that first cell cycle after recovery using the parameters that we did. We found these aberrations. So, I will leave it at that point.

DR. LEOPOLD: Did the aberations persist after the material was allowed to continue growth on earth?

DR. KRIKORIAN: That was one stupid mistake we made. We should have made the attempt to keep some of the plants alive, but of course, we didn't. We fixed them. We were working under terrible conditions at the space recovery area where there has been a windstorm. So, we were covered in white sand at White Sands, working in a trailer, rushing around like madmen trying to do all of this, but at least I think there is enough information.

DR. KAUFMAN: Did you detect any aberrations during meiosis?

DR. KRIKORIAN: We don't have any evidence on that, but there is meiotic difficulty in some of the reports on Tradescantia. That was worked out by people at Brookhaven under the late Arnold Sparrow, but, also, there is some work by the Russians, but in their case they have attributed it to temperature aberrations in the spacecraft. The temperature profiles that we have seen and which we attempted to mimic in oats and in mung and in the sunflower, we have never been able to achieve that kind of aberration, and I have consulted with other cytologists in the oat, and they say, "My God, what a mess." Whether they could recover or not is a mute point. I don't know.

DR. HEPLER: Do you see the mitotic abnormalities in carrot?

DR. KRIKORIAN: Again, at that time we did not have the cytological techniques worked out for carrot. I am happy to say we do now, but I have a feeling we probably wouldn't, you see, because the carrot for weird reasons that I cannot explain seems to be a remarkably stable organism. There is no such thing as endopolyploidy. There is no variation in the karyology of carrot. You have got to really muck them up before they do anything.

DR. ROUX: Thank you, Abe.

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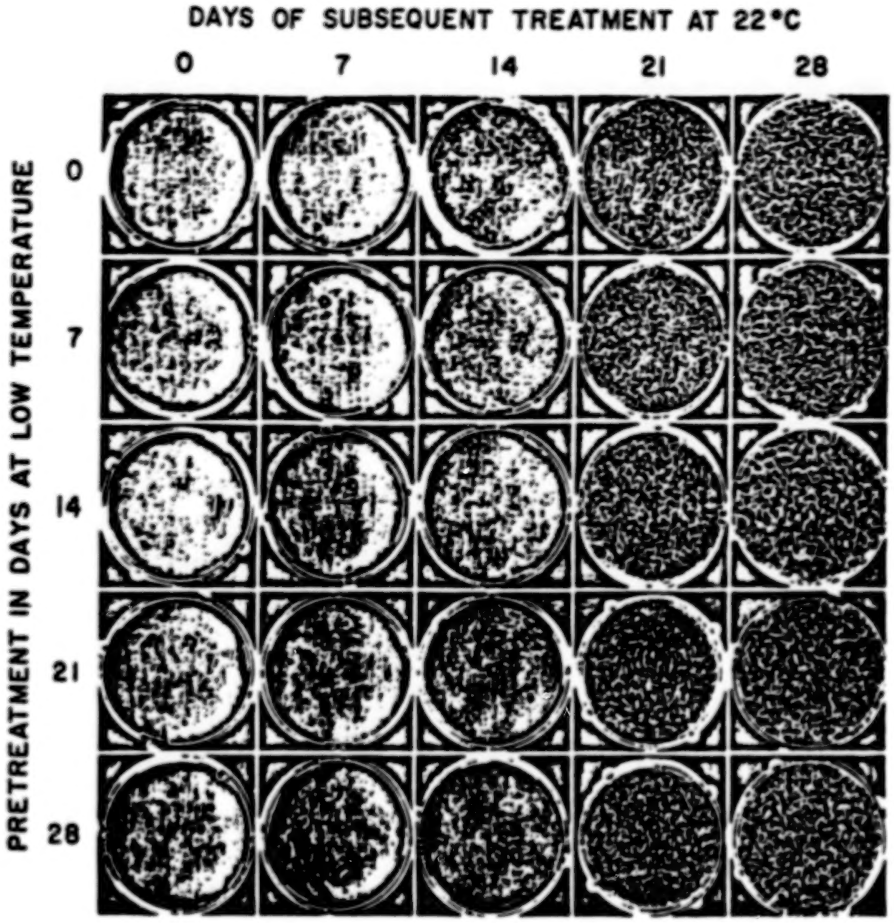
KRIKORIAN FIGURE


DAYS OF SUBSEQUENT TREATMENT AT 22°C
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7
14
21
28
PRETREATMENT IN DAYS AT LOW TEMPERATURE
0
7
14
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28

Fig. 1. Shows that morphogenesis in totipotent cells of carrot is depressed by low temperature (4°C) and this is subsequently reversed at higher temperature (22°). The growth shown on each dish was the outcome of the number of days at 4°C, as indicated on the vertical scale at the left, followed by the number of days at 22°C shown on the horizontal scale. Note that a maintained low temperature for as long as 28 days, during which growth did not occur, did not interfere with the ability of the cells to develop when they were transferred to the higher temperature (from 3).

KRIKORIAN FIGURE

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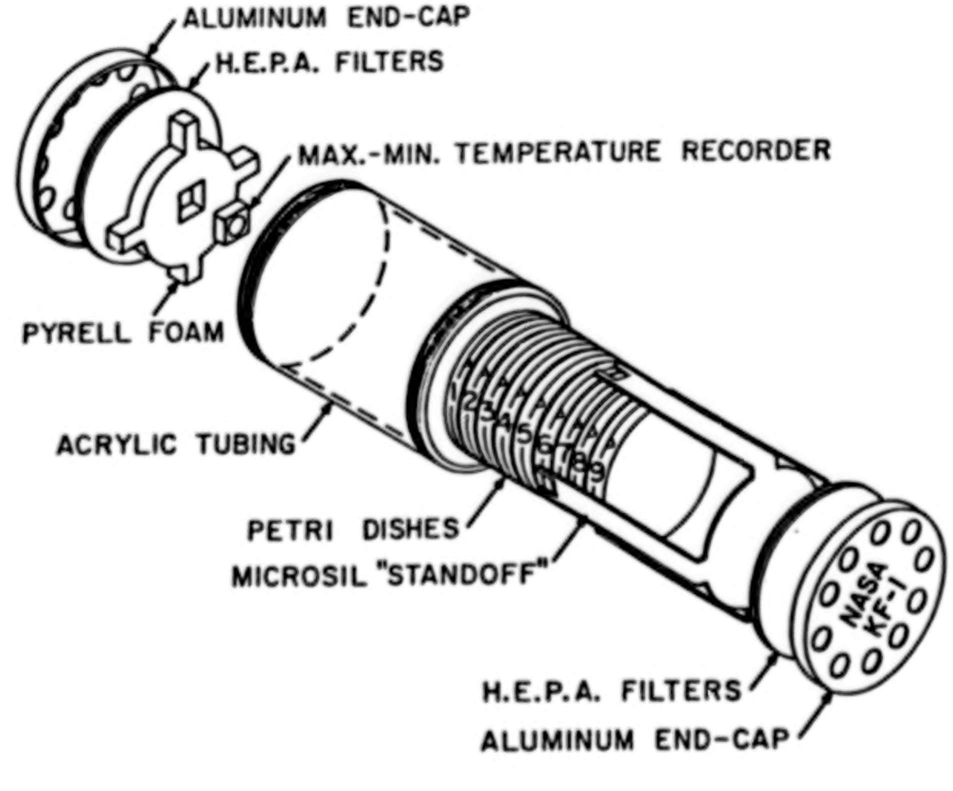
Fig. 2 Diagrammatic representation of a flight canister in disassembled perspective showing petri dishes etc. The numbers on the petri dishes represent position in the canister, 1 is at the top of the canister while 9 is at the bottom (from 3).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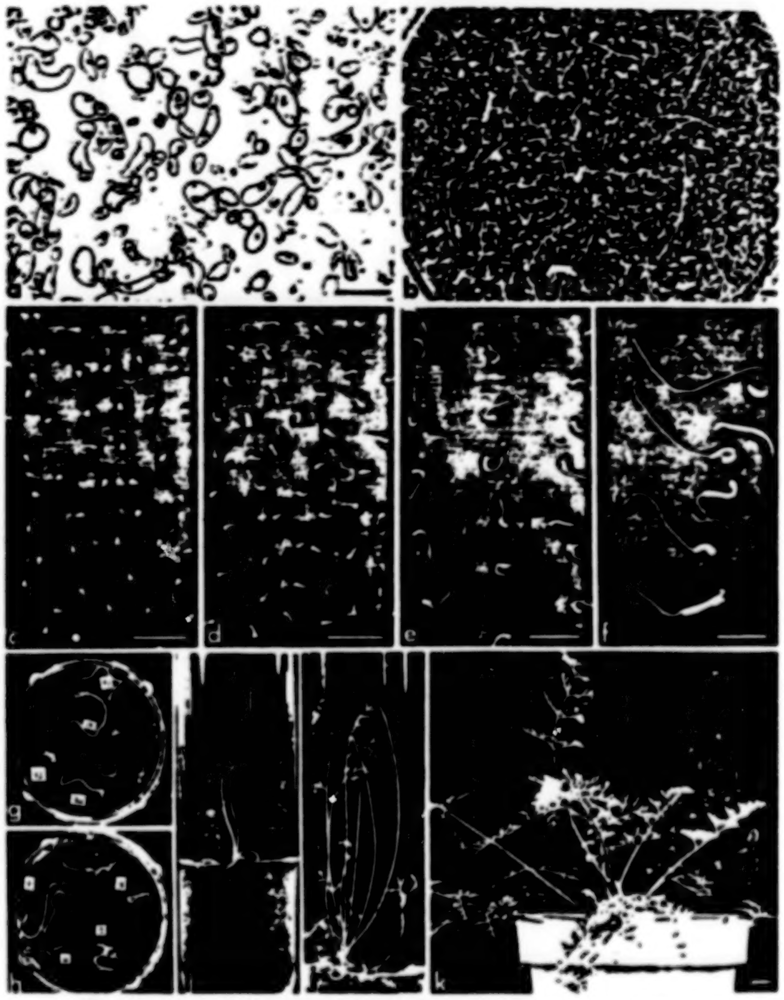
KRIKORIAN FIGURE

FIGURE #3



Development of somatic embryos from totipotent carrot cells under near zero gravity conditions and their subsequent growth into mature plants. (a) General appearance of cells from suspension culture after gradation through sieves with decreasing pore size (scale bar, 100μm). (b) Development of carrot cells into embryonic forms after 20 days in space. The low magnification (scale bar, 1 mm) and the dispersal of the profuse crop of embryos *within* as well as *on* the agar medium make it difficult to distinguish easily all the forms but typical globular, heart (c), and torpedo-shaped (d) (scale bar, 5 mm), as well as mature embryos, (e and f) (scale bars 1 cm) were present and could be aseptically isolated. Embryos at each of these stages could be removed from the dishes and further grown to maturity in successive steps; (g) and (h) show 50 mm diameter dishes each with several representative embryos that arose during flight and were transplanted to fresh medium and grown for approximately 1 month; (i) and (j) show two 35 mm diameter culture tubes with plantlets as from (g) and (h) at time of initial transfer to tube (i) and after 40 days' subsequent growth (j); (k) carrot plant reared from (i) and grown in soil for 2 months (scale bar, 1 cm.),(from 4).

KRIKORIAN FIGURE

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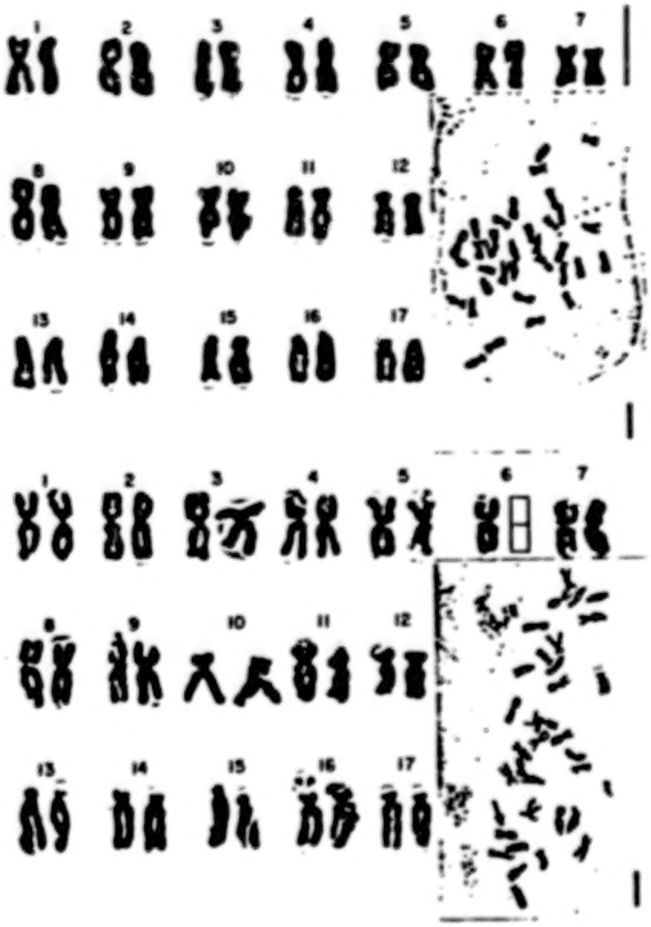

Fig. 4. Comparative karyotypes of Helianthus annuus cv. 'Teddy Bear' made root rip cells of plants exposed to hypogravity in the Reflex Bioengineering Test (RBT) of Brown and Chapman. Top panel, the inset shows a representative cell stopped in metaphase (scale bar, 5 μm). The rest of the panel shows the chromosomes from that cell cut out and rearranged as an associated diploid karyogram. Arrangement of homologous pairs is according to their centromere position and according to decreasing size. Chromosome pairs 1-7 are metacentric; 8-12 are submetacentric; 13-17 are subterminal. Lower panel, the inset shows a representative cell stopped in metaphase (scale bar, 5 μm). The rest of the panel shows the chromosomes from that cell cut out and rearranged as an associated diploid karyogram. The homologue to pair 6 (see arrow in inset) is missing. The microsatellites on pairs 9 and 16 are more readily seen in this preparation than the one above but this is not to be taken as a difference. The sole difference between the plants is that in one instance (upper panel) the chromosome complement is 2n=34, whereas the other plant is aneuploid 2n-1=33. The condition of aneuploidy in H. annuus has not been described hitherto insofar as we are aware.

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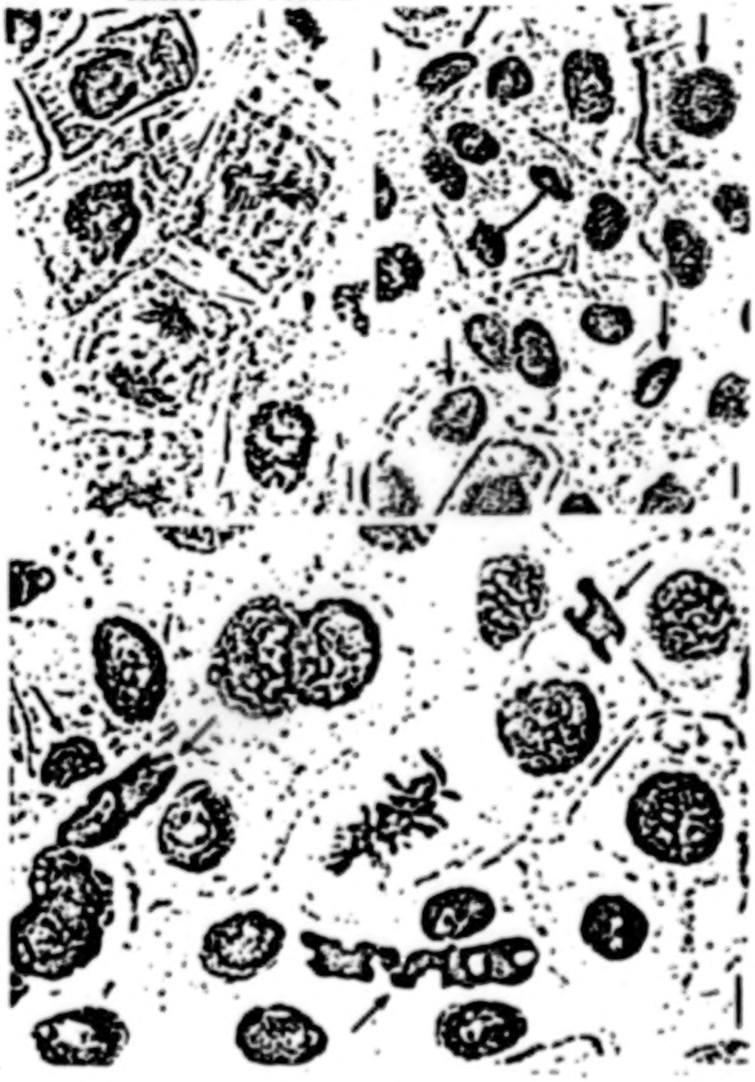

Fig. 5. Some representative division figures from root tip cells of _H. annuus_ cv. 'Teddy Bear' exposed to hypogravity in the Reflex Bioengineering Test (RBT) of Brown and Chapman. Top left panel, field showing various stages of division. Interphase metaphase, anaphase and mature cells are all visible (Scale bar, 5 μm); top right panel, field showing cells with normal active nuclei (arrows) and others in the telophase stage of division. A chromosome bridge at telophase is clearly visible. This is an extreme form of mitotic abnormality and leads to an abnormal complement and usually death (Scale bar, 5 μm). Lower panel, field of sunflower root cells showing abnormal nuclear components. These elements appear to have been "thrown" out of some of the cells. The arrows point to what has been here interpreted as progressive damage to nuclei. The two nuclei at 9 o'clock (left and right respectively) are shrunken and eroded, the material at 1 o'clock represents a still more extreme case and the string of nuclei at 6 o'clock are still more so (Scale bar 5 μm).

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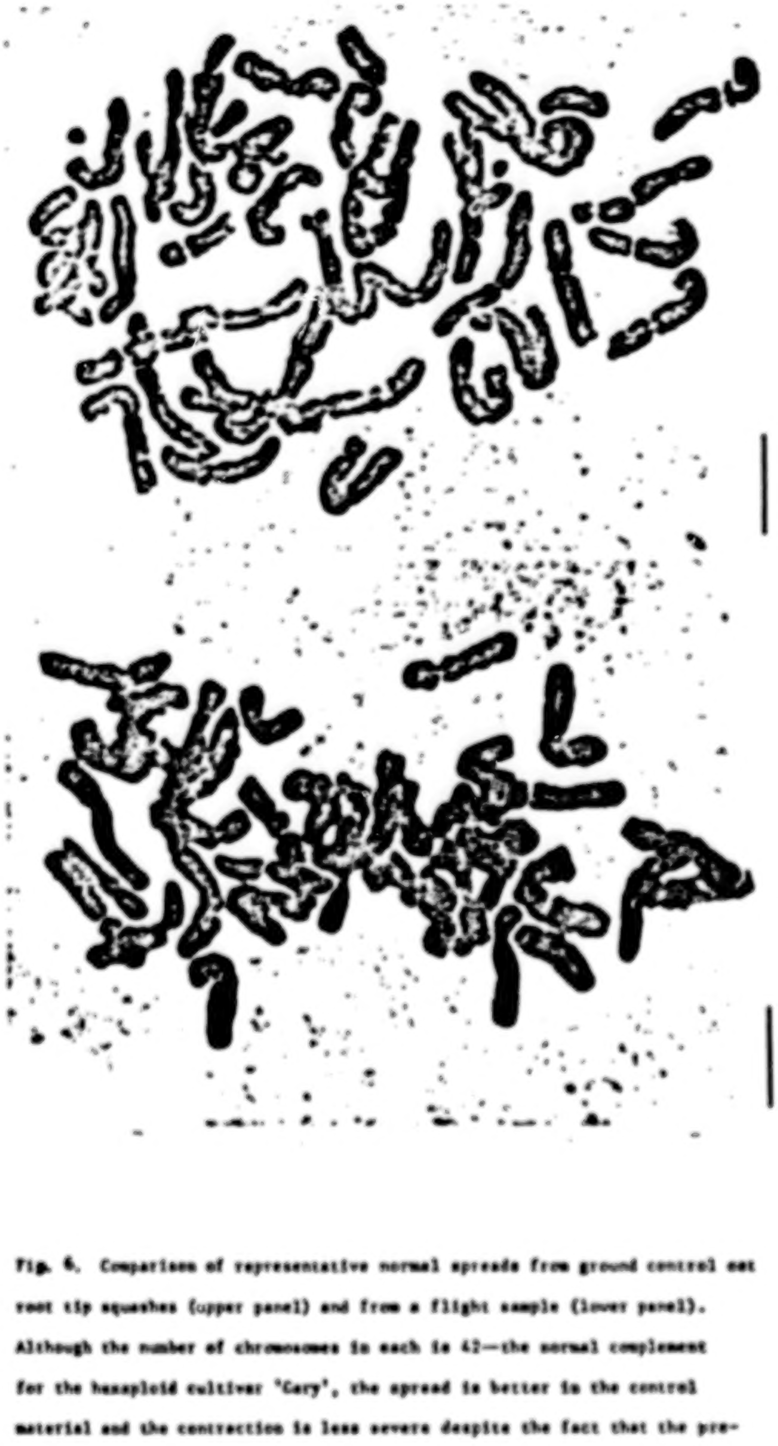

Fig. 6. Comparison of representative normal spreads from ground control oat root tip squashes (upper panel) and from a flight sample (lower panel). Although the number of chromosomes in each is 42—the normal complement for the hexaploid cultivar 'Gary', the spread is better in the control material and the contraction is less severe despite the fact that the pretreatment regimens were identical. There was a general tendency for the flight material to be more contracted; it also stained more densely. Chromosome morphology was more difficult to study as a consequence.

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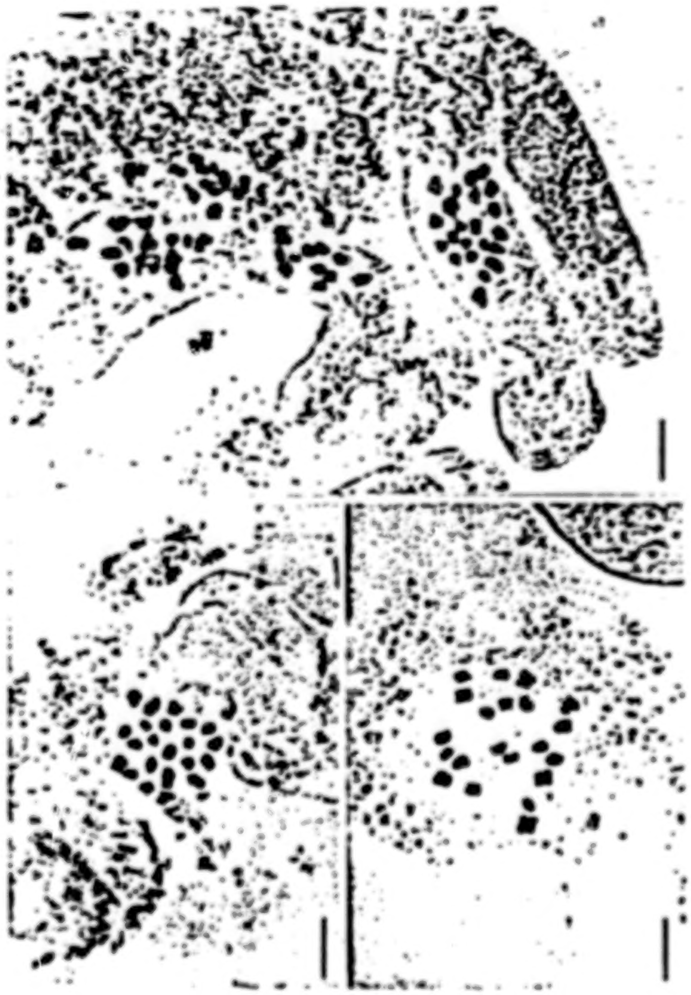

Fig. 3. Representative squash preparations from root tip cells of mung bean. Upper panel, flight sample showing five metaphase nuclei in a field of about ten cells. This sort of preparation permits one to establish that dividing cells do exist. Lower left panel, metaphase plate from a flight sample showing the full diploid complement of 2n=22 chromosomes; lower right panel, metaphase from a ground control again showing the normal complement. The chromosomes in each of these cases are too small to permit karyotype analysis but there is no evidence whatever of abnormalities—at least insofar as the techniques permit. The gross morphology of the chromosomes seems comparable. However, in general, the flight material exhibited greater chromosome contraction and a poorer spread than those nuclei from the ground controls. (Scale bar, 3 um).

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DR. ROUX: The second speaker in this short glimpse into gravitational effects on plants is Peter Kaufman from the University of Michigan. He will be speaking about gravitropic responses in the grass pulvinus: Model system for asymmetric growth.

DR. KAUFMAN: I would like to first start by telling you what the grass pulvinus is. It is really called the pseudopulvinus. A pulvinus is usually responsible for the sleep movements in leaves. So, this is truly a pseudo pulvinus that doesn't have sleep movements, but responds nicely to gravity, that is, it is the primary site in the shoot of the grass plant that shows a negative gravitropic response when it is gravi-stimulated (Figures 1 and 2). In this paper, however, we shall use the term pulvinus for the gravity responding "organ" since it is so used entrenched in the literature.

So, let us first introduce the system to you. We have these swollen joints which are actually the bases of the leaf sheaths. They represent the gravitropically sensitive pulvini of the grass shoot.

In most of the grasses that you are familiar with, such as wheat, oats, and barley, have one pulvinus at each leaf insertion site. Grasses, such as corn and bamboos, *Panicum* species, and the millets, have two pulvini, one at the leaf-sheaf base and one at the internode base just *above* the interclary meristem. The latter are large coarse grasses which during evolution have apparently developed two pulvini at each leaf insertion site to help orient the shoot when it is gravi-stimulated.

During gravity-stimulation, it is usually one or two pulvini along the shoot that respond in the so-called festucoid grasses like oat, wheat, barley, or rye. In the panicoid grasses, you may observe as many as 12 of them responding to gravity at any one time. The reason pulvini in older shoots do not respond to gravi-stimulation like you see here is that they become highly lignified and silicified, and thus, the pulvini cells are incapable of responding to gravity.

The gravity response phenomenon is shown in our next slide (compliments of the US Department of Agriculture at Beltsville) to show the relevance of this response (namely, recovery from lodging) to a food crop, sugarcane. During the response quite a number of pulvini curve upward as you can see in the photograph. Sugarcane has one of pulvinus at the sheath base and one at the internode base. So you have two pulvini that are actually responding to gravity at each joint.

SPEAKER: You sure you are not pushing it with your hand?

DR. KAUFMAN: No, not pushover! "Pushing on" now, this is an oat (*Avena*) shoot in which and you can actually lower it below the horizontal and get curvature of more than 90 degrees. We have measured as much as 120 degrees on these shoots. You can flip them upside down (180°) and they will reorient themselves and by reversing curvature.

There is an example of a festucoid grass with two pulvini responding, usually with the older one not showing as much curvature as the one that has recently been initiated. The very youngest ones will not respond to gravity, they are gravitropically incompetent to respond. Here is a panicoid grass, and you see two pulvini at each joint which respond to gravi-stimulation. You have seen this response in the panicoid grass, crabgrass, in your lawn when they come up and flower very much like this one showin in this slide.

Here is the response to gravi-stimulation in rice. These plants were treated with the hormone, gibberellin, resulting in expression of the foolish seedling disease syndrome, and because of the overgrowth of these internodes here and the leaf sheaths, the shoots have now responded to gravity. You can see that it orients the shoots to an upright position, and here is a closeup of that.

Now, the agricultural significance of this is a phenomenon called lodging, where the plants fall over due to the action of wind and/or rain or when we have used too much nitrogen. It can result in significant reductions in grain yield, particularly in rice, oats, wheat, or barley, and even sometimes with corn. In deep water rice which grows up to 10 meters in length in paddies in Bangladesh and Vietnam, the shoots show this remarkable internodal extension; then when the paddy dries during the dry season the shoot become gravi-stimulated; as a result, the shoots curve upwards via the negative gravitropic response. It is also called kneeing ability, and non-kneeing rices are genetically and economically very undesirable. So this shows you something about the significance of this phenomenon in nature. It is a way by which the plant orients itself in the vertical position. This allows for more effective seed dissemination, and also, the plants are photosynthetically more competent.

Now, let us look at the pulvinus itself. Here it is in the oat plant. This is a swollen leaf sheath base. This is leaf sheath up here, internode below, and this is the swollen pulvinus with thousands of brick-like cells on the surface. It is these cells in the pulvinus that were laid down during intercalary growth of this leaf.

Now, let us look at a scanning electron micrograph of the pulvinus. This is a secondary electron image of the pulvinus of rice and if you obtain an x-ray map for silicon over the same area, using an x-ray analyzer coupled to the scanning microscope, you see abundant silicon disposition in the sheath above and in the internode below and essentially not any above background in the pulvinus itself. So, it is silicon poor pulvini that are capable of responding to gravity. Here are comparable SEM views in another grass that has abundant hairs on the pulvinus surface. You see that the silica is mostly localized in the hairs but not in the pulvinus cells that would respond to gravity.

So, the grass pulvinus that is competent to respond to gravity is silicon - poor (1).

Not only that, but it is, also, lignin-poor (1). If you test the tissue with lignin-specific stains such as pararosanaline hydrochloride or phloroglucinol-HCl, you can detect lignin in the tissue very nicely in the sheath tissue above or in the internode tissue below. The vascular bundles that extend longitudinally through the pulvinus that are only cells that have any lignin whatsoever. In the pulvinus, this tissue here is called collenchyma. Up here, in sheath tissue above the pulvinus, are fibers. Fibers have highly lignified walls. The collenchyma, in contrast, is non-lignified. Thus, the pulvinus tissues are not only relatively unsilicified, but also are, for the most part, non-lignified in those that are competent to respond to gravity.

This is the oat leaf-sheath pulvinus. Here, in corn, you can see the leaf sheath pulvinus and the internodal pulvinus you see at a single node locus that in both cases, the pulvinus is essentially lignin poor. Lignin only is seen in a few tracheary elements in the internodal pulvinus and in the leaf sheath pulvinus. So, to summarize then in the panicoid grasses, there are two pulvini at each node, one at the base of the leaf sheath and one at the base of the internode. Both are lignin poor as you see here. In the festicoid grasses, we have a single pulvinus at each node, namely, the leaf sheath pulvinus. It has very little lignin present in it at a time when it is capable of responding to gravity.

Now, let us look at the cellular basis for the gravitropic response in the grass pulvinus. Is cell division involved? Absolutely not. The response to gravity is simply one of maximal cell elongation on this lower side and zero cell elongation on this upper side as shown in this gravi-stimulated rice shoot. This is a lateral bud. This is the internode inside. The internode is passively carried along during gravistropic curvature, although there is some cell elongation that has occurred on this lower side.

The tissue systems we are talking about in the leaf-sheath pulvinus are illustrated here. We have about 60 vascular bundles present, two rings of them, with smaller bundles in the outer ring and large bundles in inner ring. The bundles occupy about one-third of the total volume of the pulvinus. The starch statocytes are localized right here (Figure 3). I am going to show you a better picture of that when we used I_2KI, which is a specific stain for starch to show you where the statocytes are located. This is a large collenchymatous cap over a vascular bundle. This is the outer epidermal system; the inner epidermal system is here, and the vascular tissue in each bundle is shown here. The statocytes as shown with I_2KI staining, occur in the inner side of each vascular bundle. Thus, the starch statolites are localized to the inner side of the pulvinus in a ring of statocyte cells (starch-containing cells).

Here, the starch statoliths are shown in this scanning electron micrograph of a pulvinus that was cut in half. You can see these large amyloplasts which sediment within 10 minutes when you gravi-stimulate the pulvinus. Here they are shown in the process of sedimenting as shown with the TEM. When you look at their structure more carefully, they not only have a double membrane around each one, but also have a unit membrane, namely the tonplast (vascular membrane), which is carried down with them (Figure 4). We are not the only ones that have shown this. It has been shown in Germany and by a couple of other labs in this country. This observation of the tonoplast membrane coming down with the sedimenting starch statoliths is very important as far as compartmentation is concerned. Vacuoles, for example, have the main sources of gibberellin conjugates in plant cells. Larry Rappaport at University of California, Davis has recently shown this to be true. Thus, because the tonoplast membrane covers down along with the amyloplasts, it could be a very important thing both physiologically and biochemically in terms of hormone action as we shall allude to shortly.

Now, let us look at some of the physiological characteristics of the grass pulivinus system. We use an angular recording transducer to determin the kinetics of growth. We have done this on Bob Bandurski's corn seedling system. It starts with his corn coleoptils within five minutes and the whole curvature response is over within 2 hours. It is very important, if you are going to do biochemical or physiological work, to have these kinetics for curvature worked out.

In the grass pulvinus of oat it starts by 20 minutes, in barley by 15 minutes. The starch grains sediment, to the bottoms of the statocyte cells in 10 minutes, and the gravitropic response starts 5 to 10 minutes later. Upward curvature of the pulvinus of oat or barley proceeds at about 1.5

degrees per hour for about 48 hours (Figure 5). This response is temperature dependent, and for these kinetics, we used 30 C. In Bob's corn coleoptile system, the upward curvature goes much faster, about 1.3° per minute.

In the grass pulvinus system, the cells elongate maximally on the lower side, up through the organ. They elongate progressively to a lesser state; and at the top. By making epidermal peels at the top, middle, and base, you can see this graded asymmetry in cell elongation.

If you peel off the pulvinus epidermis, then gravi-stimulate it, you do not nullify the gravitropic response, contrary to what our friends Firn and Digby say in Britain. With barley, oats, and corn, we have peeled off the pulvinus epidermis, keep the pulvini in a humid environment, and start the transducer; all of them respond gravitropically. You only lose about 5 degrees curvature, probably because of the trauma of removing the epidermis, but the pulvini are still competent to respond. So the locus of perception is not in the epidermis *per se*, contrary to what our colleagues have claimed.

In the pulvinus, the collenchyma tissue forms large strands that surround the vascular bundles (2). Let me show you the collenchyma response during gravi-stimultion. Here are non-gravi-stimulated and gravity stimulated pulvini (after 48 hours). The collenchyma cells in the gravi-stimulated pulvinus have elongated extensively on the lower side, up to ten times their original length (2).

SPEAKER: Those are single cells?

DR. KAUFMAN: Yes, they are single cells. In their cell walls, the thin regions correspond to pits based on microscopy studies. The thin regions may be sites where auxin or some other hormone may be diffusing out and causing a localized thinning of the cell wall.

To summarize then, we have zero cell elongation response at the top to maximal at the bottom of the pulvinus. We want to emphasize that there is an a gradient of increasing cell elongation response from top to bottom in vascular tissue, collechyma, and parenchyma (3). So, in any physiological or biochemical work it is important not to just analyze tops and bottoms of gravi-stimulated organs, but to look at sections at several levels, to be able to establish that there are gradients, say, for example, with calcium or with auxin and gibberellins or with wall-loosening enzymes; really, whatever kind of chemistry you are going to go after, you have to consider this fact that there is zero response here at the top, a graded response between, and maximal at the bottom. So that you cannot say that all the action is going on in the base half and none in the top half. All the cells respond to gravi-stimulation except the uppermost ones.

Now, we have a very potent tool in terms of the celulose synthesis inhibitor, DCBN. Lincoln Tais suggested that we try it in the grass pulvinus system. It turns out to greatly reduce the gravitropic response. This first one is control, upright control. This second one is gravi-stimulated one. This third one was gravi-stimulated but couldn't respond; it just swelled up; it was treated with colchicine. This fourth one also showed little curvature and also swelled up considerably (Figure 6); it shows us that cellulose synthesis is necessary for gravitropic curavature.

SPEAKER: May we have the inhibitor again?

DR. KAUFMAN: 2,6-dichlorobenzonitrile, DCBN. It can depress gravitropic curvature as much as 50 percent in 48 hours in oat pulvini.

Look what it does to the cells, as seen by fluorescence microscopy. I want you to start focusing now on some of these walls out here. See what has happened to them? You see these striking radial cellular swellings right here (Figure 7). The cells have tried to respond to gravity; instead of elongating, they have become greatly swollen. On their radial walls, the swollen portions do not consist of cellulose but other wall polysaccharides such as pectins and hemicellulose.

The swellings on the radial walls, are now shown in TEM views. If you plot the frequency of those swellings with respect to the gravity vector here, you see the majority of them occur in cells on the bottom side (4). They first appear in a statocytes in the time course series, then later in other cells of the pulvinus. So, therein is cellular asymmetry with respect to the effects of DCBN on the radial and not the tangential walls.

Now, let us examine the transduction phase, namely, the time of establishment of hormonal asymmetry that occurs in this and other grass pulvini. You can apply gibberellin, right here, place the shoot portions with their pulvini here under several glass slides, and supply them with 0.1 molar sucrose (they need substrate to respond), put them in the dark, and this is the curvature response you get after 48 hours with sucrose, and here it is with sucrose plus gibberellin. You get the famous internodal extension also with the gibberellin treatment, but you can also accelerate pulvinus curvature about 40 percent.

Now, one thing interesting about the GA work is as follows: In collaboration with Dick Pharis at Univerisity of Calgory, we applied the hormone, that is, we fed corn with tritiated GA_{20} (^3H-GA_{20}), one of the precursor metabolites in GA biosynthesis in this plant. Here ^3H-GA_{20} is applied in micro-drops around and just below the pulvinus. The pulvini are kept vertical as controls. We found that you can get a fairly large amount of label here in the sheath pulvinus and an even larger amount of label in the internodal pulvinus; presumably it is able to come in from right here and move up into this site here.

Now, if you apply ^3H-GA_{20} here at the top, and you gravity stimulate the plants for 12 hours, you see this kind of a picture. No isotope appears on the lower side, either in the internodal pulvinus or the sheath pulvinus. The label all resides at the top. You can apply radioactive GA at the bottom of the pulvinus, and the label all remains at the bottom. It does not move up. So, corn pulvinus system is incapable of transporting GA when the pulvini are gravity stimulated. We found the same thing as Malcolm Wilkins has found with C_{14} IAA. It also is totally incapable of moving from top to bottom in a gravity stimulated pulvinus. This is not morphological top, but really the side of the pulvinus, now in top position when the pulvinus is in gravity stimulated.

Now, as for the native GAs in this system, we have looked at all these parts of the oat plant. These N's are nodes. These are internodes here, next to last leaves (NTLL), last leaf (LL), the influorescence, and the root system. We find that the bulk of the GAs are in the influorescence, but the node being the second richest source (5).

There is as much as 1850 picograms of GA per influorescence and about 46 picograms per node pair. So, the node is a very rich storage source of native GAs. The primary native GA in oat is GA_3, but we have just recently isolated (with Dick Pharis and his group) GA_4 and A_7.

Now, when one gravi-stimulates the oat plant for 24 hours and get about 30 degrees curvature here for the pulvini and then separate them into tops and bottoms, you find that the bulk of the, free, active GAs (A4, A7 and

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the A3) occur in the bottom half as shown by the yellow bars in the histogram, whereas the top halves have much, much less. This asymmetry was detected by means of silicic acid partition chromatography and the dwarf vice ('Tan-ginbezu') bioassay, If you pre-feed with tritiated GA_4 (a precursor) and then chase the acid metabolites and the conjugates, you find that the conjugates pile up in the top halves, either in the internode or the pulvinus, and the acidic GA_4, A_7, amd A_3 pile in the bottom halves. So our active GAs are on the lower side, and the storage conjugated GA's pile up on the upper side (6).

Since the pulvinus cannot transport GA from top to bottom in the gravi-stimulated pulvinus, how do you establish such an asymmetry? One possible mechanism is that GAs are released preferentially from their conjugates, mostly the glucoside. We thus propose that GAs are released from their ester glucosides to a greater extent on the lower side than on the upper side in the gravi-stimulated pulvini.

Now, the auxin story. I am going to show you a wigwam here. This is a series of oat shoots, and this is what auxin does when you apply it unilaterally (on the outsides of the pulvini) after 24 hours: the wigwam closes up. This is after 48 hours. So, it is very responsive to auxin applied exogenously and you can see that you can stimulate the pulvini curvature very markedly in this system with IAA.

Then you can analyze for endogenous auxin (free IAA) by separating these pulvini into lefts and rights (upright controls) and tops and bottoms (gravi-stimulated to 30°) and collect 2000 halves (10 gram samples) over as short a time as possible. It is sheer agony to do that, but you have to do it to find out. Even more ideally you fractionate this pulvinus tissue into several fragments rather than just top and bottom halves. We use Bob Bandurski, Aga Schultz and Jerry Cohen's procedure which is very sensitive. It is the double internal standards that allow you to do a very accurate accounting of the free IAA in the system and in the intacts you have 60 nanograms free IAA per gram of dry weight of tissue in the intact, upright pulvinus, and in the lefts and rights of upright pulvini, 70 nanograms free IAA per gram dry weight - great difference. But lo and behold, in the gravity stimulated after 24 hours (about 30 degrees curvature), you get essentially almost a 1:3 ratio for free IAA, top versus bottom, and seven times more total free IAA than in the gravi-stimulated pulvini than in the uprights.

This system is also incapable of transporting auxin from top to bottom during the gravity response. So how do you get this asymmetry? Again we have postulated that it is possible that there is a release of auxin from either its inositol ester conjugate or from, more likely here, the peptidyl conjugate of IAA with release occuring to a greater extent on the lower side than the upper. Alternatively, greater synthesis of IAA coiuld occur in the lower side than the upper side.

At the present time we are doing the kinetics on this to find out when this asymmetry is established in relation to when the curvature is generated and the changes that occur in the conjugates, both for the auxin and the GAs.

Now, finally, we have the cellular asymmetry. We have already found that there is about three times more active cellulase activity in this bottom side than in the upper side. We have found by using the appropriate inhibitors that protein synthesis is necessary for the gravitropic response; also DNA synthesis and RNA synthesis. So, you have got a lot of metabolism going on. At the present time we are looking at this system in its different loci to determine the levels of activity of key wall-loosening enzymes, (cellulase, pectinase, hemicellulose or arabinoxylanase). We are also analyzing the cell walls to look at their polysaccharide composition, namely, the pectins,(polygalacturinans), the arabinoxylans, and the cellulose content. We are also analyzing the wall protein, and recently, have done some work on electrophoresing the proteins. We find that during gravity stimulation, we lose two or three protein bands from the lower halves but not from the upper halves, this is a new finding.

The other thing we are going to do is to go after the proteins that are increased in amount and find out which ones they are. The key candidate marker ones would be peroxidase which is an enzyme that is able to deconjugate GA from its ester glucoside, cellulase, pectinase, and arabinoxylinase. These are all candidate key marker enzymes that we would like to go after and determine electrophoretically how they change during gravity stimulation. And finally, we want to get at the actual mechanism for the perception when the starch statoliths fall to the bottom side.

We have postulated that this wall, this outer tangential wall, OTW, is different from the inner tangential wall(ITW), and when the statoliths fall on the inner one you do not get a response, whereas here you do when they fall on the outer one. There is something different either biochemically or morphologically about the OTW as compared to the ITW. It could explain the asymmetry which we are getting in growth. Since the statolith falls and carries with it the tonoplast (6), there is a possibility that bound IAA conjugates could reside on the plasma membrane and the deconjugating enzyme, say, a peptidase or one of the deconjugating enzymes that releases IAA from its inositol ester conjugate (e.g., peroxidose) could be brought down to this compartment and result in release of the free IAA which could move laterally but not basipitally and cause cell wall loosening here. I think we should stop right there.

DR. ROUX: We have a few minutes for questions.

DR. DELA FUENTE: What happens after there is a curvature and then you invert this?

DR. KAUFMAN: About a 10-minute lag it reverses curvature; it is still going down when you turn it upside down. Let us put it this way.

Now, after the pulvinus has shown a gravitropic response, we are going to turn it upside down, and about 10 minutes it is still going down this way. Then it slowly comes back up and completely reverses curvature, but it has a memory. We talked about memory systems, and this one has one. It is still sensing the original gravity vector of this position, and it will continue down based on the transducer experiments. Then it rights itself and comes all the way back up. So those cells that didn't respond at the top are capable of responding. If it only takes 10 minutes to do that, how do you account for all the asymmetry in hormones or other growth factors, say, calcium for example, when you can get that reversible curvature? We have tried the calcium on this system and found no asymmetry in our hands yet, but we are still working on it.

DR. PICKARD: It is very interesting that the vacuole membrane should be dragged down. Do you think that is rather universal. Recently Heathcote, for example, made a big point that this should not happen. Obviously he is not doing EM techniques but he claims that with the statoliths moving, very conspicuously moving around the ends.

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DR. KAUFMAN: I wouldn't be surprised if this happens in roots, in root caps or in other systems. I mean we have not looked at other systems. We have only looked at several grasses,but that is as far as we have gone.

DR. PICKARD: Sometimes in the root cap this has fallen, is very small, and there would be very little opportunity to fall through,around or near a vacuole.

DR. KAUFMAN: But these pulvinus cells have about 90 percent vacuole, these ones here. So they are really huge. So, they have got to really move through those vacuoles to get down and root cap cells may be chock full of vacuoles.

SPEAKER: They are very small vacuoles.

DR. KAUFMAN: Very small vacuoles, right, and you may have some problems with that.

DR. HEPLER: There is a topology problem here. I would be very much more surprised if they did not take the vacuole, tonoplasts with them. I think that they are compelled to stay in the cytoplasmic space, and so that is probably like a strand of cytoplasm that moves down with it and pinches off and then re-fuses at the bottom which you showed in your diagram here. So, the amyloplast stays in that cytoplasmic space. I would be very surprised if it broke out of that into the vacuole.

DR. KAUFMAN: Yes.

DR. KRIKORIAN: Peter, what do you see in the lazy mutants?

DR. KAUFMAN: Lazy, (see Figure 8) mutants - okay. The statoliths fall in lazy maize, the la/la double recessive. They fall in the normal time. We don't know about the hormone asymmetries. We are now just doing the GA's this week, and the auxin situation will be examined later. We have just planted the corn for that, but there may be an impairment in, say, release mechanism of hormones form its conjugate. As for a hormone transport mechanism, we cannot hope for anything there because there is some lesion in auxin or gibberellin transport.

DR.KRIKORIAN: But it is fairly far on, it seems.

DR. KAUFMAN: It is very way down the line because everything else happens earlier.

DR. THOMPSON: There are so many things that happen there, and I am sure you are looking for some of the primary events. Is it possible to retard some of those things from happening by just the physical restraint of bending?

DR. KAUFMAN: Frank Salisbury has done that, and they (cells in Xanthium shoots) bloat up very much like the DCBN results I showed. Then you release the shoots, and then they take off. The thing tries but it, of course, cannot do it. By the way, in the grass shoot it has a righting mechanism anytime it leans slightly. It corrects itself. The next morning you see it. It leans this way; it corrects itself. So, it is a beautiful correcting mechanism to right the shoot at any time during its development.

Kaufman

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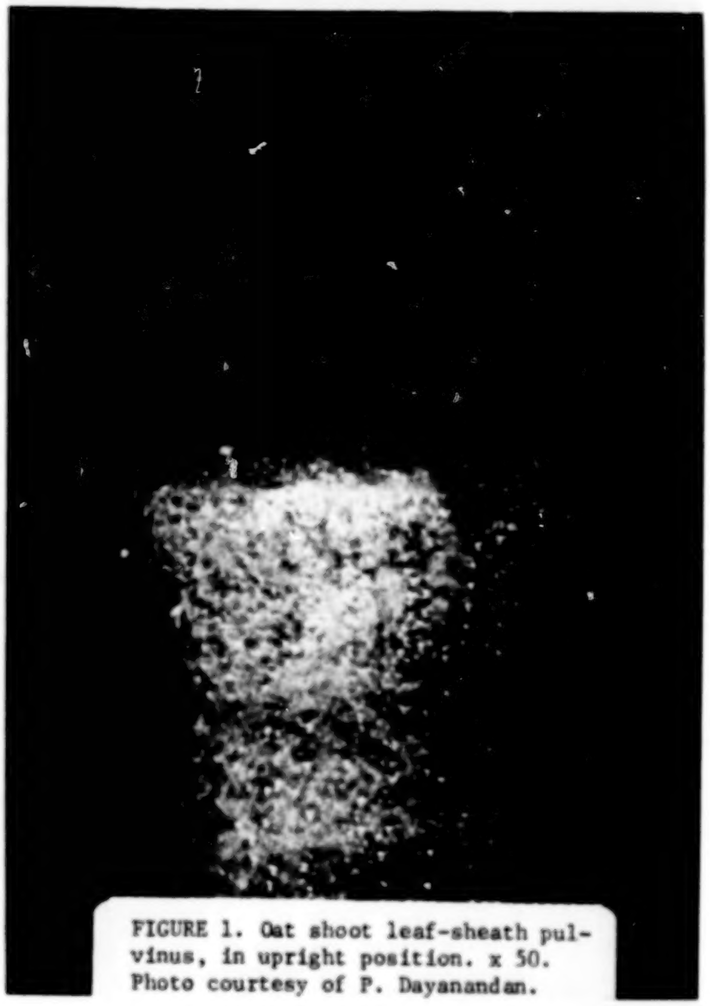

FIGURE 1. Oat shoot leaf-sheath pulvinus, in upright position. x 50. Photo courtesy of P. Dayanandan.

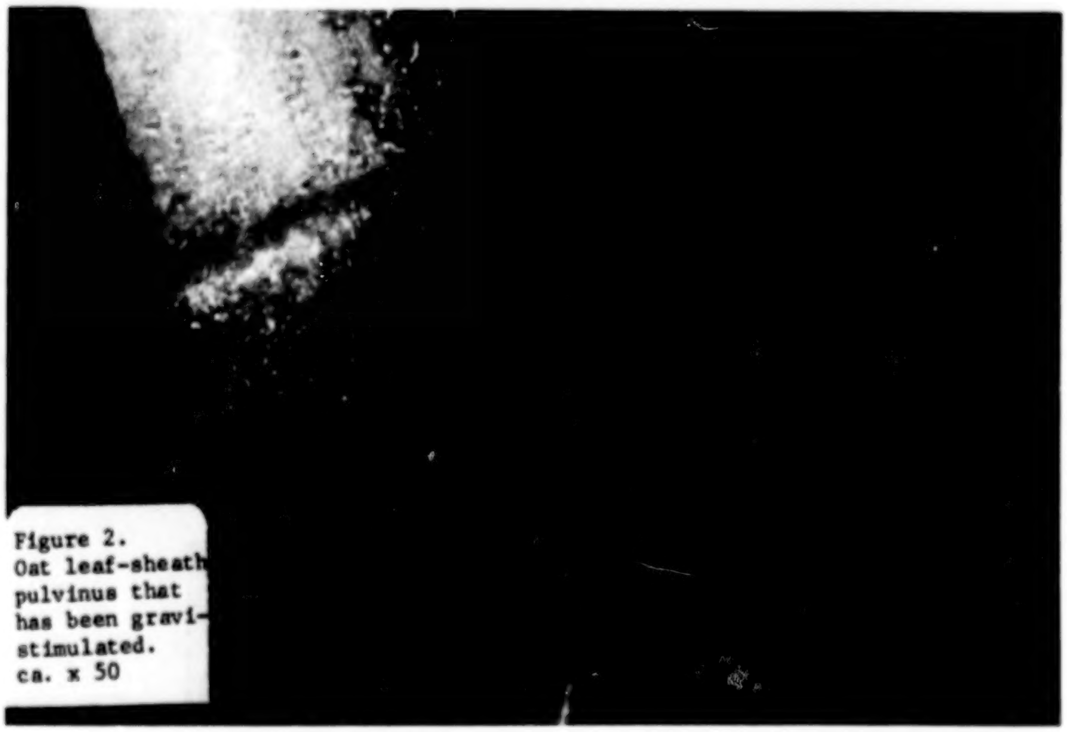

Figure 2.
Oat leaf-sheath pulvinus that has been gravistimulated.
ca. x 50

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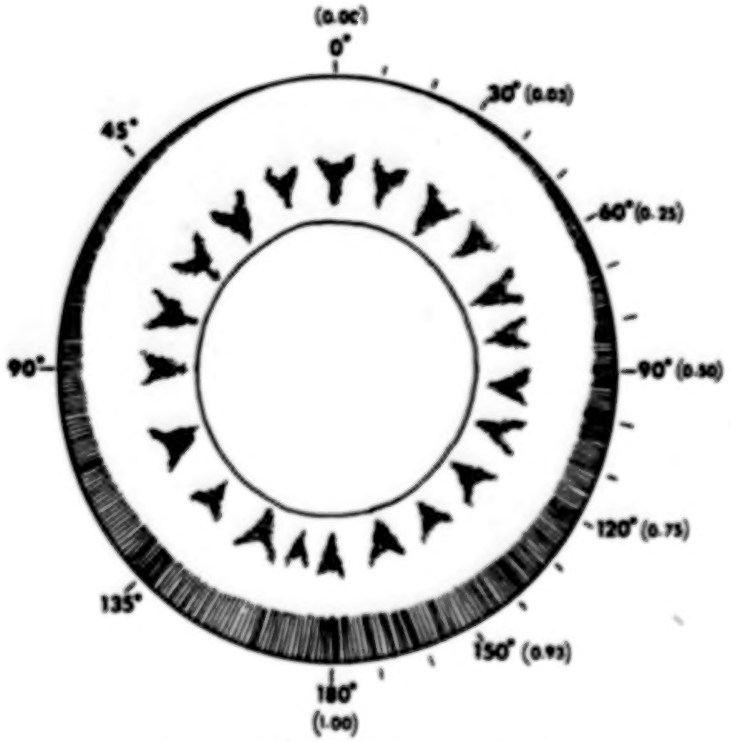
(0.00)
0°
30° (0.03)
45°
60° (0.25)
90°
90° (0.50)
120° (0.75)
135°
150° (0.93)
180°
(1.00)

GRAVITY-SENSITIVITY PROFILE

Figure 3: Transverse section of a typical leaf-sheath pulvinus, studied in shoots of oat (Avena) and barley (Hordeum), showing the location of the starch statoliths (denoted by stippled regions).

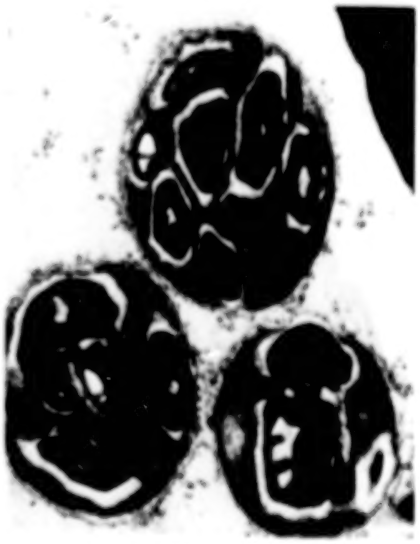

Figure 4. Tonoplast around amyloplast in Avena cells. (x 24,000)

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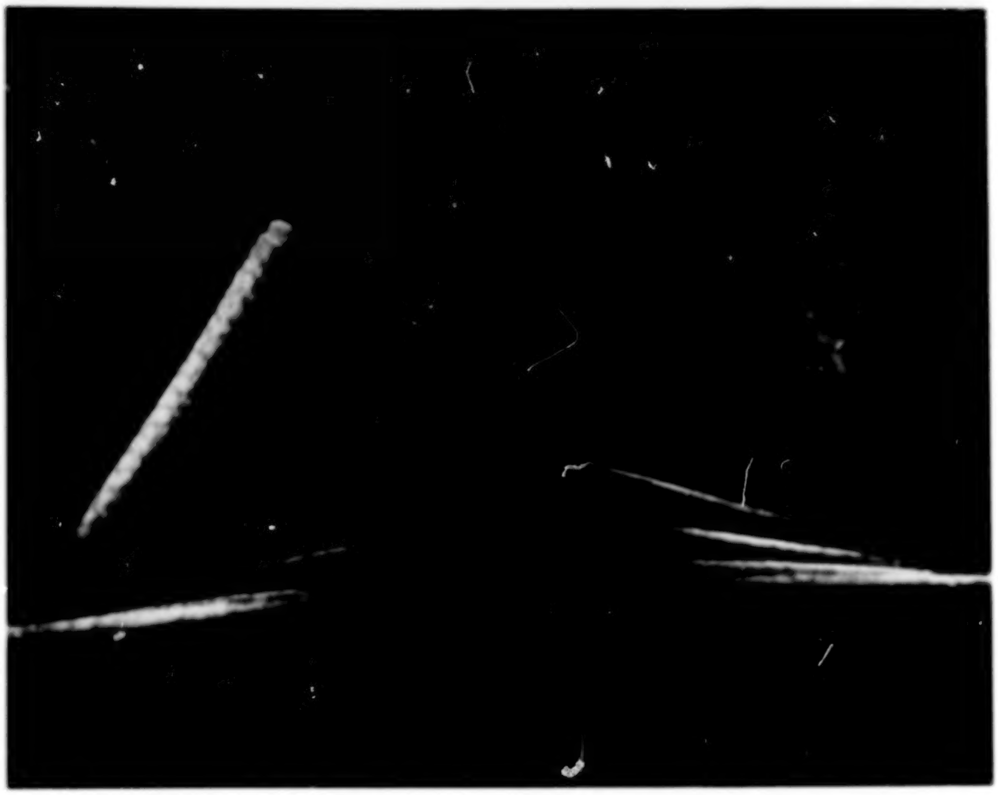

Figure 5. Time lapse photographs (taken every 3 hr) of the negative gravitropic curvature response in oat leaf-sheath pulvini in excised shoot portions which were base-held (left) and tip-held (rt.) Photo courtesy of P. Dayanandan

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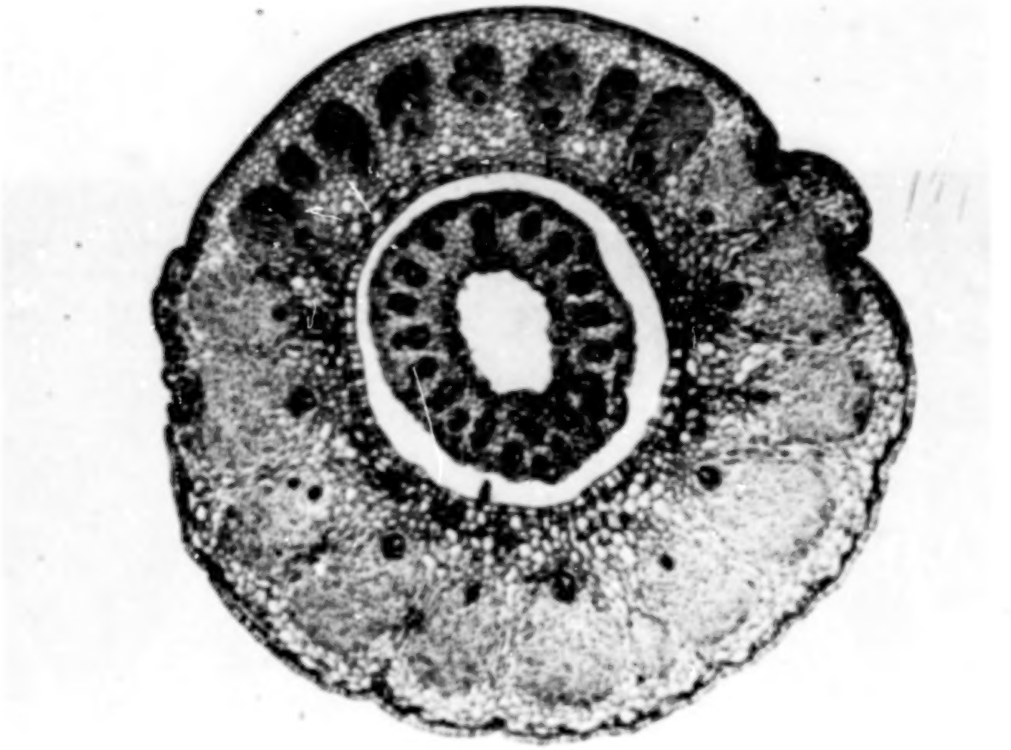

FIGURE 6. Barley 24 hrs. gravistimulated + DCBN. x 50. (Photo by Dayanandan)

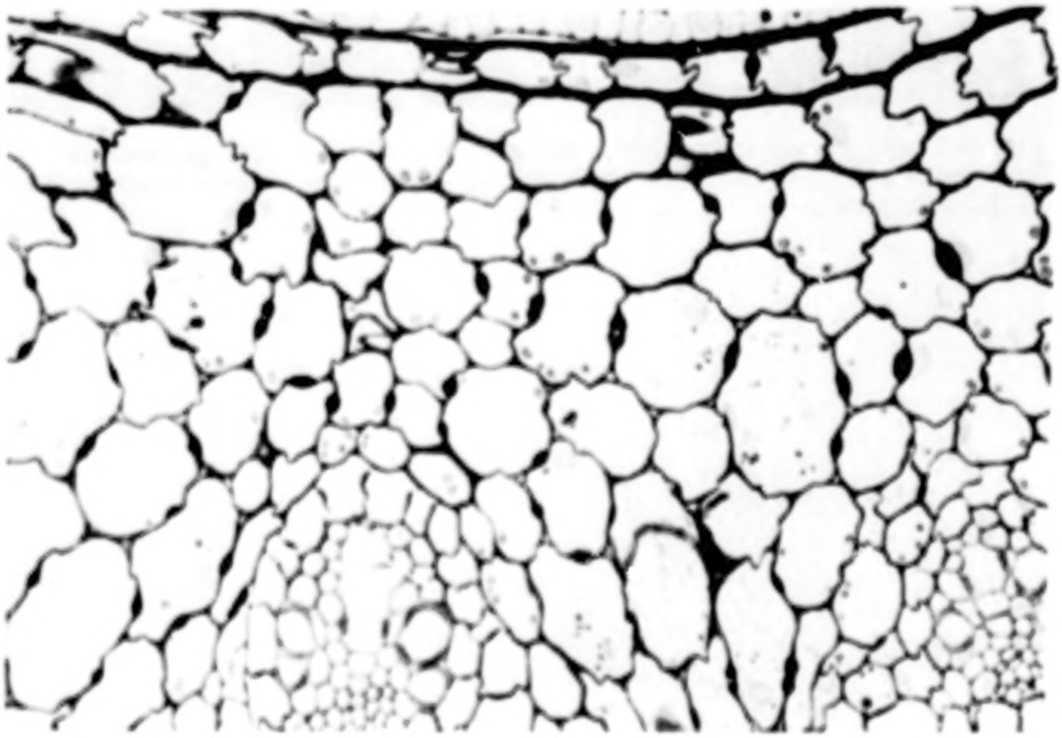

FIGURE 7. Barley + DCBN, as in Fig. 6. Lower half of pulvinus. x 750. (Photo by Dayanandan)

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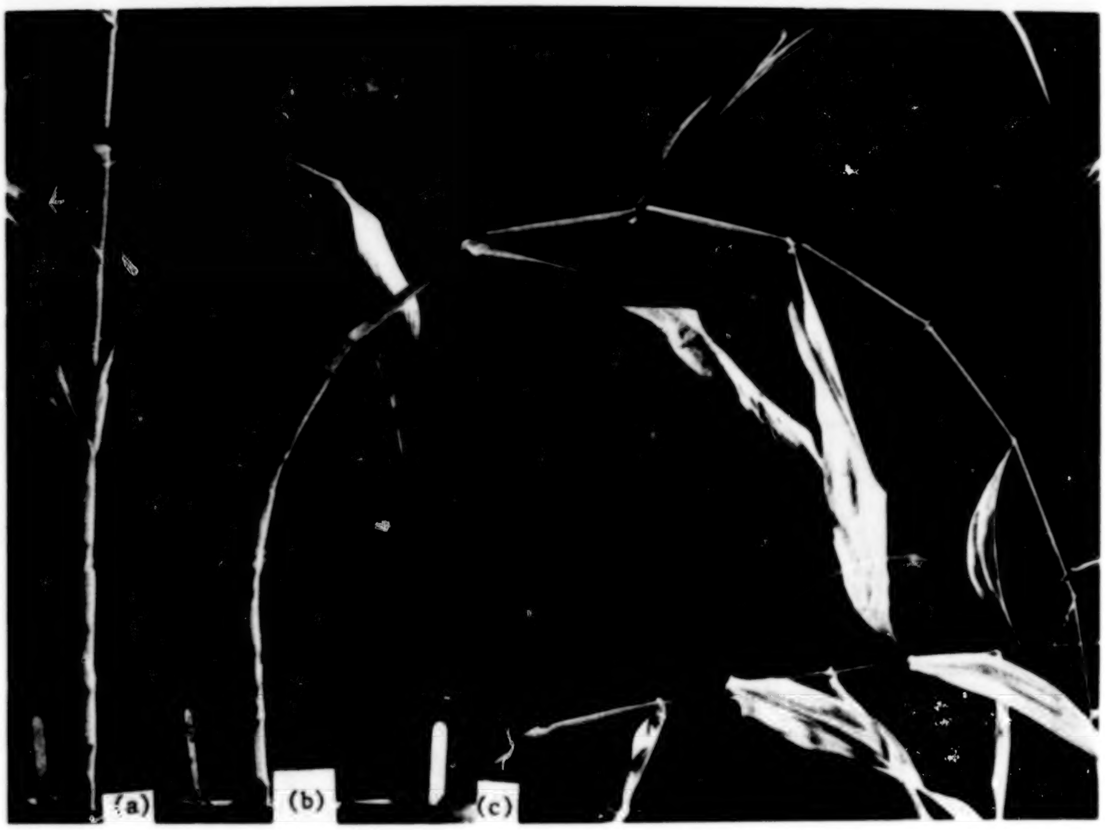

FIGURE 8. (a) Normal corn--shoots upright.(b) and (c) "Lazy" mutants of corn whose shoots became ageotropic during development. Photo courtesy of P. Dayanandan.

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DR. ROUX: The next speaker will be Emily Holton from NASA Ames Research Center, and her topic will be bone and calcium alterations during spaceflight.

Emily?

DR. HOLTON: I am going to divide this talk into three parts. The first part will simply be descriptive information on why we are interested in calcium and bone. The second will be some actual flight data we accumulated over the last six or seven years, and I will describe the Cosmos series which Dr. Krikorian just alluded to. Finally, I would like to philosophize about what the data might mean and present a working theory that tends to say that gravity may be very important in some of these calcium-mediated responses.

Table 1 is simply to point out something that has been long known, that as one increases in size on the face of the earth, the percent of the body mass which is represented by the skeleton increases. This suggests that skeletal size is loading dependent, and so it is not surprising that if one is to remove that load, for example. By going into space, that indeed one might expect some alterations to occur.

Even the popular press has noted that when one unloads the skeleton, one loses mass. In fact not only does the press use a word which has become rather common -- osteoporosis; it goes on to define what osteoporosis is: washing out of calcium. So, here we have two very important terms, the first one being osteoporosis and we like to go a little further and say that what we are really interested in could be termed space disuse osteoporosis and a second term, calcium, which of course is found and stored primarily in the skeleton in mammalian structures.

So, it is not surprising that when we started sending people into space, information indicated that calcium balance was negative; that is, they were taking in less calcium than they were putting out. Obviously, one can project that calcium loss is probably coming from the skeleton.

We knew by the early seventies that in astronauts there was an increase in urinary calcium and possibly, with time, an increase in fecal calcium. The system is losing calcium. Within 84 days in space astronauts appeared to have a detectable loss of skeletal mass in the heel bone. Measurements of the radius and ulna did not suggest any loss of that particular mass. This result led to the conclusion that the major defect was in the weight-bearing bones. Now, with what we have since learned, one wonders if this indeed is a correct interpretation.

When it was obvious that the manned spaceflight program was drawing to a close for the near future in 1973 when Skylab was flown, we were invited by the Soviets to participate in a series of missions. These missions were to involve primarily rats. The spacecraft used was an 8-foot diameter sphere. It was launched by Voshtok rockets, and circled the earth for approximately three weeks before it then returned to earth. Now, as has been pointed out, one of the problems with flights of this type is that you only have preflight and postflight measurements. One has to control for all the perturbations that may not be realted to the exposure to near weightlessness but, indeed, to the insults of launch and re-entry.

When one works in one's own lab, communication difficulties can arise. So, imagine what it is like to have your experiment done in a country where a different language is spoken, and your experiment is conducted 9000 miles away, and you have really no choice in selection of the animals or the age of the animals, and you want to get as much information as is possible.

The Soviets tend to do things in groups of fives (Table 2) We were not invovled with the first two missions. The United States participated in the last three flights: Cosmos 782, 936, and 1129. It is interesting that the Soviets consecutively number their missions, so you can see how many rockets they launched in about a six-year period. The launches which we participated in were two years apart. All missions lasted somewhat less than three weeks, and the orbital parameters were very similar.

We had to write down procedures in a stepwise fashion because, if we did not go to the Soviet Union to teach the techniques for our experiment, the the Soviets would translate our directions and process the materials accordingly. In cases with complicated procedures we did send the US scientists over to demonstrate. The recovery of the Cosmos biosatellites occurred in Siberia. The Soviets had a van which they drove up to the satellite and they would deploy a mobile lab from this van, and on the site would dissect half of the flight animals. They had a crackerjack team. They would guillotine an animal. The head would go down one side, the body down the other, and within 20 minutes they had stripped everything out of that animal that you could possibly want.

As I pointed out, the Kosmos spacecraft is an 8-foot diameter sphere. Rats were housed individually in cages which were about 3 by 8 inches. Each group of five cages had its own food and water supply. The Cosmos 936; included an on-board 1 G centrifuge, to see if the perturbations noted in previous space flights could be corrected. One of the real problems with the centifuge was that it had a very short radius, and the animals were not happy with this very short arm on the centrifuge.

The protocol for 1129 included male Wistar rats. In the previous two flights they had been 63 days of age and weighed approximately 190 grams at the beginning of flight. This flight was delayed for 20 days, and the animals were 20 days older and 100 grams heavier when they were launched. In addition to the flight animals, there were two ground-based controls. The flight control group was sealed in the backup satellite. This group really simulated the flight in terms of caging, restraint and feeding cycle, and light-dark cycle. The vivarium controls, on the other hand, were group housed and kept in the vivarium.

There were two test periods. The flight period was approximately 3 weeks in duration. At the end of the flight period, half the animals were killed and tissues removed. The other half of the animals were allowed to recover for approximately 3-4 weeks.

The animals were labeled with a milligram per kilogram of demeclocycline for our particular experiment where we wanted to look at bone formation rates. Animals were injected 3 days prior to flight because of flight constraints and at various times following flight to outline the flight period of bone versus the bone formed postflight.

On 782 and 936, I collaborated with Dr. David Baylink. NASA knew that 1129 was going to be the last rat mission in the Soviet projected experiments, so we decided to make as much use of the tissue as possible to get much basic information on the changes that might be occurring. We involved laboratories across the country, and the data I am going to present is a compilation not only of my own laboratory findings, but, also, the findings contributed by many of these other investigators.

First, I would like to tell you about the negative data because there is very little. The first piece of negative information was from the incisor; this work was done by Dr. David Simmons at Washington University in St. Louis. He could find no alteration in dentinogenesis or mineralization of the incisors of these young growing rats. He told me recently, that they have done microprobe analysis of the incisor that was formed at the end of the flight period, and it seems there may be a defect similar to what we have seen in some of the other bone tissues.

Bone mass is a function of two processes, formation and resorption; formation is putting bone down; resorption is taking bone away. Prior to our first flight we had theorized, based on calcium kinetic information and information from immobilized animals, that we were probably going to see an increase in resorption. Much to our amazement, this did not occur.

The first measure of bone resorption was simply the medullary area at the tibia-fibular junction in these animals. The medullary area (Figure 1) can increase only by resorption. When you give a tremendous bone resorbing stimulus to a rat the size of the medullary area increases. So, our first crack at resorption was a very gross one, and whether we looked at the tibia or the humerus we could see no significant difference in the medullary area in the flight group versus either of the controls. The vertical bars represent plus or minus one standard deviation.

Initially we said there is no gross change. On Cosmos 1129, Dr. Cris Cann used stable isotope tracers. I am sure all of you know that calcium exists as a multiplicity of stable isotopes (Table 3), and these are natural markers in bone.

If one replaces all the calcium isotopes with just a single isotope, for example, calcium 40, in the diet, then one knows that the only source of the isotopes which are removed from the diet is bone. The only way this particular isotope will get into the blood or out into the feces is through bone resorption. For this experiment, animals were fed a diet solely of calcium 40. Dr Cann found that in flight versus flight controls (Table 4), the total body net resorption if anything is decreased. However, if one normalizes for turnover, there probably is no change in total body resorption.

So, the animals were not losing bone mass by increasing their resorption rate. However, if one looks at formation, one finds some really striking differences. The sites we looked at were the tibia-fibular junction and the humerus just below the ridge. If one does any sort of histomorphometry it certainly makes your data much tighter if you can find an anatomical landmark and using an anatomical landmark, such as the junction of the tibia and fibula or a ridge, the data scatter is much, much less than if you do midshaft or other portions of the bone.

On Cosmos 1129, we decided to look at the trabecular bone and the growth plate area. This became quite important because there may be preferential problems in trabecular, versus the cortical bone. Also, trabecular spicules stick into the marrow cavity and they have a much larger surface-to-volume ratio than does cortical bone. We were astounded to find indications of a cessation of formation. Figure 2 shows cross sections from rat tibia. They were taken from animals that were killed at the end of the recovery period. You can see what we have termed an arrest line. This arrest line demarcates what would have been the periosteal surface at the end of flight. Prior to our initial flight on Cosmos 782 we had never seen a cement line of this magnitude in any animals.

In Figure 3, you can see the tetracycline label. Label one is that given just before flight. Label two is the label given in the postflight period, and you can see visually that the amount of bone formed during the flight period in this flight animal was significantly less than the amount formed during the postflight period. It is also interesting that the arrest line coincides with the second tetracycline label, and in fact, was almost directly superimposed on the label. .

We are quite sure that the arrest line is not an artifact or a reversal line. A reversal line is found in many bones, and it occurs when resorption reverses to formation. It is a very uneven line, and it stains for acid phosphatase activity which indicates remnants of osteoclasts which are trapped when resorption ceases and formation begins. The arrest line, unlike the reversal line is a straight, distinct demarcation. It does not stain for enzyme activity. Continuing work is being done on this line by Dr. Russell Turner in South Carolina, and he has found it is a hypomineralized line. Unlike many cement lines which are hypermineralized areas this one appears to never mineralize. There is something very strange about the matrix that is laid down or maturing during the period that these animals are in flight.

How consistent was this suppression of formation from animal to animal? Regardless of which flight rats -- whether it was the first, second or third flight -- flight rats formed less bone during flight than they did in the postflight period (Figure 4). So, it was a very consistent finding. In fact, if one looks at bone formation versus the control group (Table 5), you can see that even though the numbers or the formation rate varied from flight to flight the percent change is very consistent and essentially we find a 40 percent suppression of formation rate. We have never been able to duplicate the extensive arrest line that we have seen in these flight animals in growth experiments.

In the last flight, we not only looked at cortical bone, but also at trabecular bone. A basal group sacrificed at launch gave some indication parameters of trabecular bone mass at this time. Much to our surprise, suppression of the tabular mass in flight rats didn't become significant actually until the end of the postflight period (Figure 5). Unlike cortical bone which recovered very quickly following flight, trabecular bone was much more sluggish. The deviation is much greater in trabecular measurements, but the trend is there, and seemed to be reflected (also in) vertebral strength. I will show you some of that data in just a minute.

Coincident with this decrease in trabecular bone mass (Figure 6) was a tremendous increase in fat in the same area. However, you can see that (marrow fat) returned to normal very quickly. In osteoporosis in humans there, also, seems to be an invasion of fat into areas where trabecular bone is lost.

I alluded to the fact that the trabecular bone loss seemed to reflect itself in vertebral strength (Figure 7). Leon Kazarian at Wright-Patterson Air Force Base did some crushing strength on vertebrae and found that, regardless of the column position the strength of the vertebra was weaker in flight animals as compared to control groups. Vertebral strength in the animals allowed to recover following flight returned toward, but not to, control levels by 29 days postflight (Figure 8). Long bones (femurs) were fractured by Dr. Dan Spangler at Washington University, Seattle; bones from the flight group were much weaker immediately following flight, but in the postflight period not only seemed to recover but possibly overshoot. So, the long bone, the femur recovered very quickly, whereas the vertebra which is primarily trabecular bone seemed to be more profoundly affected by this short exposure to a near weightless environment.

I said that the theory was put forward following manned flight, that the flight effects were limited to the weight-bearing bones. We found in 1129 that the humerus was affected almost identically to the tibia. Also, Dr. David Simmons at Washington University, St. Louis, looked at the mandible, and found no difference in total mineral or total hydroxyproline. But, density gradient centrifugation showed mineral, and hydroxyproline piling up in the lowest gradient, and there was a loss of it in the highest density fraction. Figure 9 is for calcium, but the other parameters are very similar. This information suggests that the bone is not maturing, that it is staying in a less mature form than one would have expected as compared with either control group. The formation and mineralization process is not proceeding normally.

Dr. Gene Roberts at University of Pacific Dental School in San Francisco has a bone cell kinetic technique model in periodontal ligament. By determining nuclear volume (Figure 10) of cells in the periodontal ligament he can distinguish populations of cells that he has termed progenitor cells, preosteoblasts and osteoblasts. The population less than 135 cubic microns he classifies as progenitor cells, while the population above that are preosteoblasts which divide to form osteoblasts.

He found a significant suppression of preosteoblast but not progenitor cells. This information suggests suppression of direction from osteoprogenitor populations to the preosteoblast cell line. One last piece of information comes from Roland Baron and his collaborators at Yale University (Figure 11). This very unexpected and very interesting finding occured in his remodeling system in the rat mandibular molars. They usually measure three different sites. However, he found such bad scatter in the flight group he had to dissect his data. He found that if you looked at those areas bounded with muscle versus those without muscle that indeed during flight the bone which was not connected to muscle showed a much greater suppression of formation than did those areas which indeed were connected to muscle. Muscle did not alter calcification in controls. Muscle may play a compensatory role, and inhibit somewhat the response that one sees to gravity. Since Baron's findings we have looked at tibial bone and at those areas where muscle is attached versus those that weren't and find essentially the same thing.

These findings are in jaw. So, dramatic effects occur in jaw as well as long bones or vertebra. When gravity is not present, most bones in the body are affected by this unique environment.

In summary (Figure 12), the incisor may take longer to show an effect than bones. Bone resorption is not changed. Periosteal bone growth decreases greatly and may cease as indicated by a hypomineralized growth arrest line. Alveolar bone growth decreases, particularly where no muscle is attached. Trabecular bone volume decreases and does not seem to return to normal as readily as coritcal periosteal bone growth. Fat content dramatically increases in the metaphysis.

Bone strength is significantly decreased in both femur and vertebra. Mandibular collagen maturation mineralization seems to be suppressed. We have indications that preosteoblast cells are depleted;

progenitor cells are not being stimulated to mature. Finally, Nello Pace and Arthur Smith and collaborators at Berkeley in conjunction with some of the Soviet investigators analyzed total body composition and found about a 20 percent decrease in total body calcium, and a significant loss of extracellular fluid.

The skeleton provides structural support, and I think most of us would wonder whether these effects are due to lack of load,. The body doesn't need the structure. So, it is logically not forming more until it gets to a new steady state where it, indeed, needs more skeletal mass. However, one must also think of the skeleton in terms of the calcium reservoir. If you lose skeletal mass what is this calcium loss going to do to the body or to other physiological systems? If one takes a look at those systems which are adpting during flight, and I call it physiological adaptation versus pathological change (Table 7) since the defects are reversible, changes are associated with vestibular -- cardiovascular, renal, bone, and muscle systems and red blood cell shape -- systems that require calcium for proper control and functioning.

Philosophizing with one of my collaborators at Ames, Lelia Coyne, we put together a scheme (Figure 13). I point out that it is hypothetical, and reflects our bias. We know in near weightlessness or exposure to flight certain sensory stimuli that are altered. We have an unloading of the total system, and we have an alteration in the gravity gradient. We have a magnitude change, as well as a vector or directional change. Some aspects can be simulated on the ground, either by bedrest or by somehow partially unloading the system, but at 1 G it is impossible to eliminate gravity. We have a question mark for other gravity factors over and above unloading of systems and a change in direction of the vector. Biochemical mechanisms such as calcium modulated systems, and this probably should say, "Stimulus response coupling" might then induce certain physiological alterations by either stimulating some systems or suppressing coupling in others causing both static and dynamic changes. We found decreases in bone mass, muscle mass, loss of extracellular fluid and alterations in red blood cell shape. Dynamic changes could include timing, fluxes, distribution, gradients, etc. These physiological alterations might cause systematic expressions including space motion sickness, (I think Dr. Ross will talk about the vestibular problems tomorrow morning) orthostatic hypotension which is noted when one returns into a normal gravity environment, behavioral changes which have been noticed, altered stimulus responses, space anemia, red blood cell changes and other perturbations which we don't know about. Figure 13 addresses primarily mammalian systems, but I really think you can see where plants might fit into such a system.

One of my interests is evolution, particularly evolution of bone. To project what multiple generations might look like in space, it helps to figure out how long it has taken us to evolve at 1 G. It certainly has not occurred overnight.

Several interesting papers have been published in the last few years. Lowenstam and Margulis (1) came out with a very interesting hypothesis. When they looked at various organisms it became apparent that the dynamic or most critical event necessary for eukaryotes to evolve may have been the evolution of the calmodulin system. So one begins to wonder whether indeed the evolution of the calmodulin family was the critical event that allowed organisms to grow and multiply as multicellular organisms in the face of a 1 G environment. If, indeed, gravity played a role in the evolution of the calmodulin family one wonders then what might happen to this system after multiple generations in a weightless environment without a gravity stimulation.

Thank you.

DR. KEEFE: I have forgotten the data, tensile strength in the bones, up, down?

DR. HOLTON: Down.

DR. LEOPOLD: How can loading be sensed? Would it be in some part of the brain system or would it be in the bone itself, the physical application to the structure?

DR. HOLTON: Probably both. Some people have projected that there is a piezoelectric effect and are now looking at electrical effects on bone. But then again it may involve the nervous system, blood supply and other things.

DR. LEOPOLD: If it were really just a weight application to the skeleton, then you would expect things like the leg bones and the vertebrae to feel it most and show the biggest response. The fact that the mandibles show such a nice response is such a queer response.

DR. KEEFE: But you have to keep in mind the distinction that you showed, the area with muscle, versus the area without muscle. With muscle you are having a steady state tone contraction, if nothing else is altered under conditions of low gravity, no longer weight bearing. Muscles are no longer helping you stand upright necessarily, and you could have differences as well in the physiological application.

DR. ROSS: The jaw is very important. If you destroy the fifth nerve on both sides, the jaw stays open because the function in the muscle mass is to close the mandible, I think that is a very important difference between those parts that have muscle.

DR. HOLTON: I think it was a big surprise to all the people looking at jaws. They thought they would find nothing, but they thought negative data would be important, and the tissue was available.

DR. ROSS: Especially in rats.

DR. HOLTON: Yes, that is right.

DR. WATTERSON: In these same animals was there any kind of profile or nitrogen balance studies?

DR. HOLTON: There is, also, a loss of nitrogen which is not significant.

I would tend to be very cautious in interpreting the endocrine profiles because you have re-entry, At the time the blood is taken, the system is already readapting. Also you have a tremendous corticosterone output in these animals because they have re-entered. Sometimes it took six hours to get to the animals, get them out of the spacecraft, and get them back to the tent. They have not done a lot of radioimmunoassays or actual hormone measurements. Adrenal weight is up, but is probably related to re-entry (2, 3). They did look at C cells in the thyroid. The C cells, if anything seem to be suppressed, but they very quickly hypersecrete once they are on the ground. These are gross observations, and I tend to cautiously interpret the data until we have samples taken during flight.

DR. WATTERSON: Is there any change in acid growth hormone levels or response postflight, preflight, to a nitrogen bolus?

DR. HOLTON: That has never been done.

The Soviets are sending up two primates and looking at some parameters, but they are not allowing many invasive measurements and the animals are not to be sacrificed. So, it is going to be trying to interpret non-invasive measurements.

HOLTON REFERENCES

1. Lowenstam, H. A. and L. Margulis. 1980. Biosystems 12: 27-41.

2. Plakhuta-Plakatina, G. I. 1979. Space Biol. Med. 13: 113-118.

3. Plakhuta-Plakatina, G. I. 1980. Space Biol. Med. 14: 41-46.

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HOLTON TABLES AND FIGURES

Table 1. Increases in Body Size and Relative Skeletal Size

ANIMAL	BODY MASS	SKELETON, % BODY MASS
Mouse, wren	20-30g	8
Dog, goose	5kg	13-14
Man	75kg	17-18

Table 2. SOVIET BIOLOGICAL SATELLITE MISSIONS

MISSION PARAMETERS	COSMOS-605	COSMOS-609	COSMOS-782*	COSMOS-936*	COSMOS-1129*
Launch Date	31 Oct '73	22 Oct '74	25 Nov '75	3 Aug '77	25 Sept '79
Recovery Date	22 Nov '73	12 Nov '74	15 Dec '75	22 Aug '77	14 Oct '79
Mission Length	22 Days	20.5 Days	19.5 Days	18.5 Days	18.5 Days
Period of Revolution	90 Min.	89.6 Min.	90.5 Min.	90.7 Min.	90.5 Min.
Apogee	424 Km. (261 Mi.)	389 Km. (241 Mi.)	405 Km. (251 Mi.)	419 Km. (260 Mi.)	394 Km. (244 Mi.)
Perigee	221 Km. (135 Mi.)	223 Km. (137 Mi.)	226 Km. (140 Mi.)	224 Km. (139 Mi.)	226 Km. (140 Mi.)
Orbital Inclination	68.2°	68.2°	62.8°	62.8°	68.2°

HOLTON TABLES AND FIGURES

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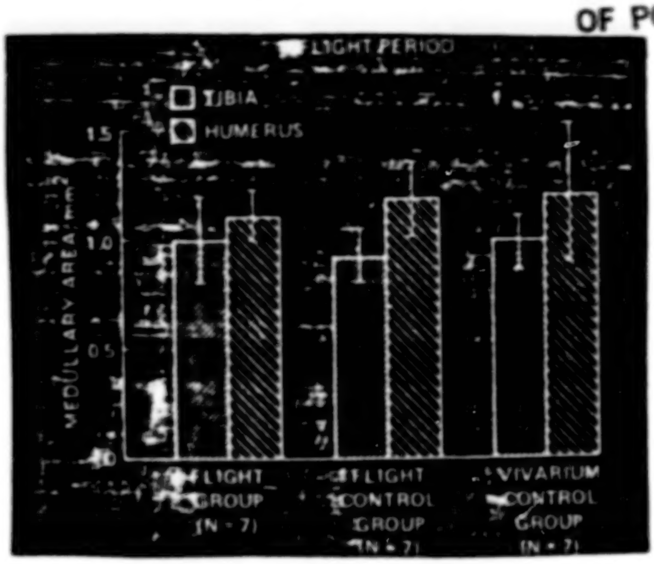
Figure 1.



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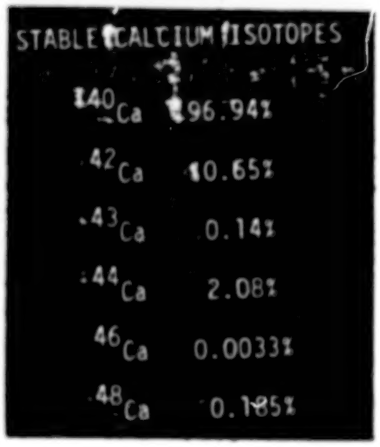

STABLE CALCIUM ISOTOPES	
^{40}Ca	96.94%
^{42}Ca	0.65%
^{43}Ca	0.14%
^{44}Ca	2.08%
^{46}Ca	0.0033%
^{48}Ca	0.185%

Table 4. RESULTS OF CONTINUOUS TRACER ADMINISTRATION STUDIES

PARAMETER	**FLIGHT**	**CONTROL**
E, R+0	**0.690 ± 0.089**	**0.675 ± 0.085**
$(^{48}Ca/Ca)_F$, R-2	**0.159 ± 0.011**	**0.157 ± 0.006**
μg ^{48}Ca Excreted/Day	**29.0 ± 3.1**	**37.4 ± 3.2**
RESORPTION, mg Ca/Day	**15.7 ± 1.5**	**20.2 ± 1.7**

E = bone resorption expressed as the fraction of exchangeable calcium from bone
F = fecal excretion

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HOLTON TABLES AND FIGURES

Figure 2.

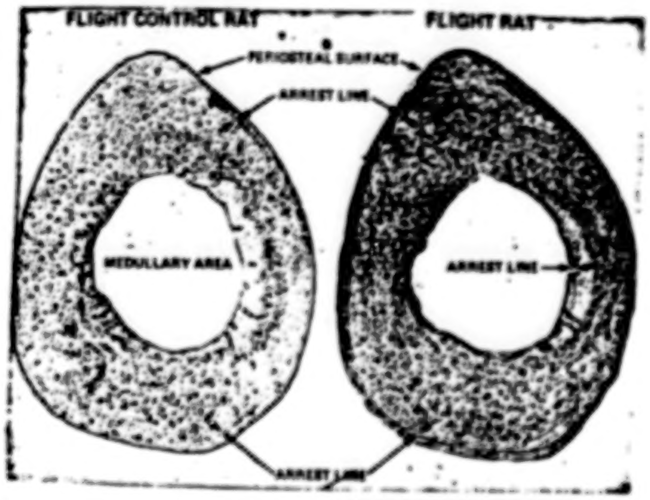

FLIGHT CONTROL RAT
FLIGHT RAT
PERIOSTEAL SURFACE
ARREST LINE
MEDULLARY AREA
ARREST LINE
ARREST LINE

Figure 3.

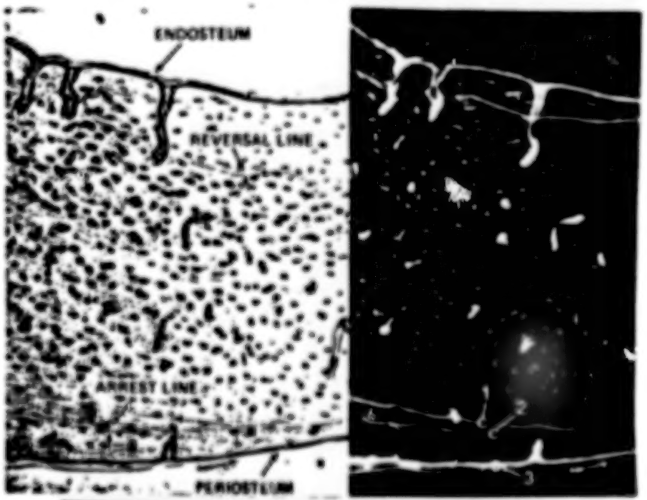
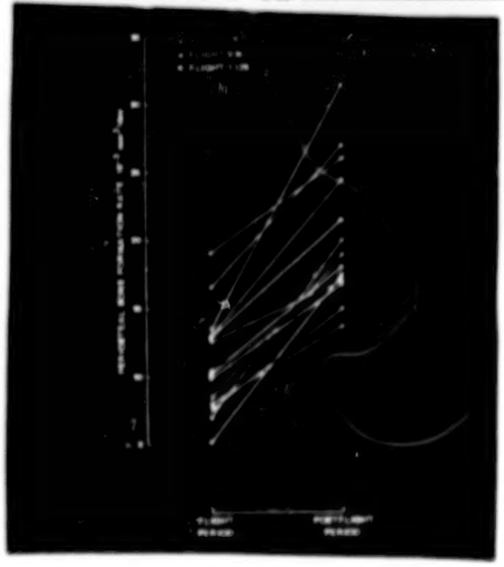

ENDOSTEUM
REVERSAL LINE
ARREST LINE
PERIOSTEUM

Figure 4.



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HOLTON TABLES AND FIGURES

Table 5.

PERIOSTEAL BONE FORMATION RATE
$10^{-3} mm^3/day$

	COSMOS 782	COSMOS 936	COSMOS 1129
FLIGHT	9.4 ± 2.8	16.0 ± 3.2	10.0 ± 2.1
FLIGHT CONTROL	15.8 ± 1.5	25.6 ± 2.9	17.9 ± 2.7
% DECREASE	40	37	44
VIVARIUM CONTROL	16.0 ± 1.4	26.2 ± 3.7	22.6 ± 4.7
% DECREASE	41	39	56

Figure 5.

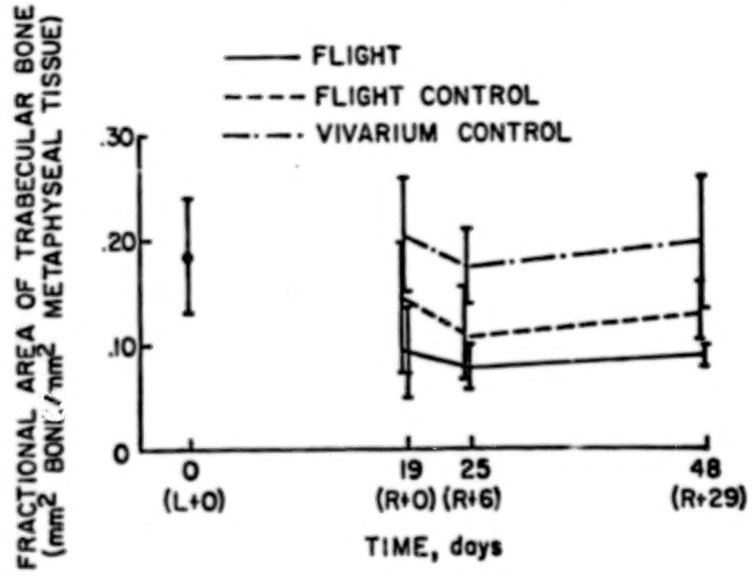

Figure 6.

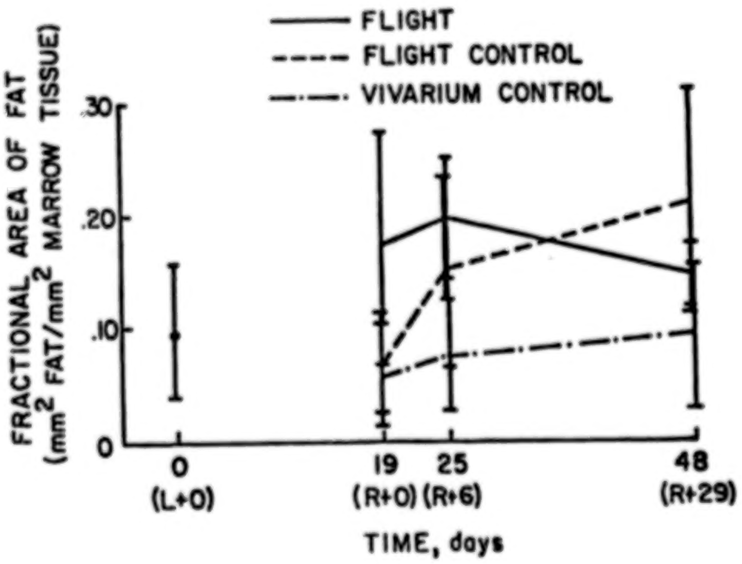

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Figure 7.

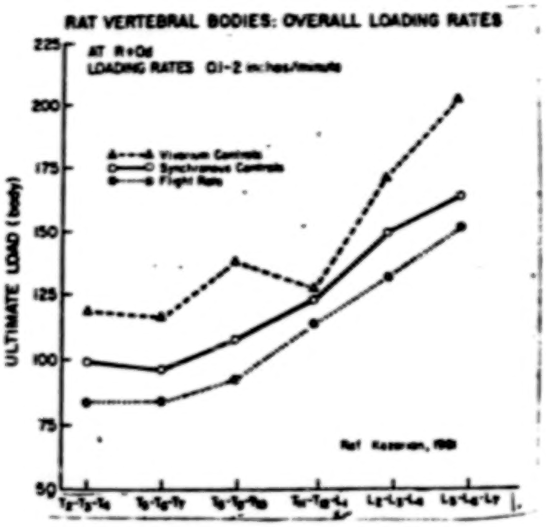


Figure 8.

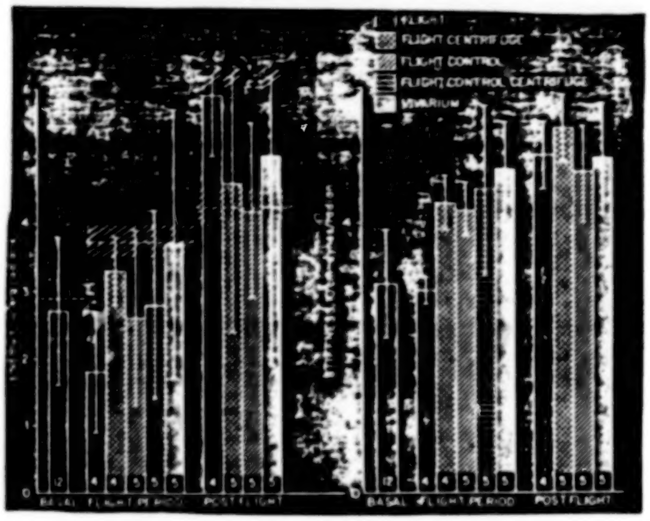
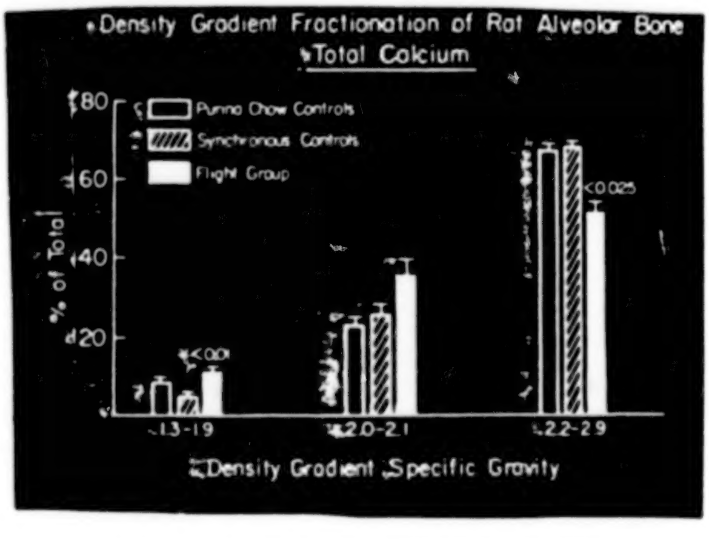


Figure 9.



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HOLTON TABLES AND FIGURES

Figure 10.

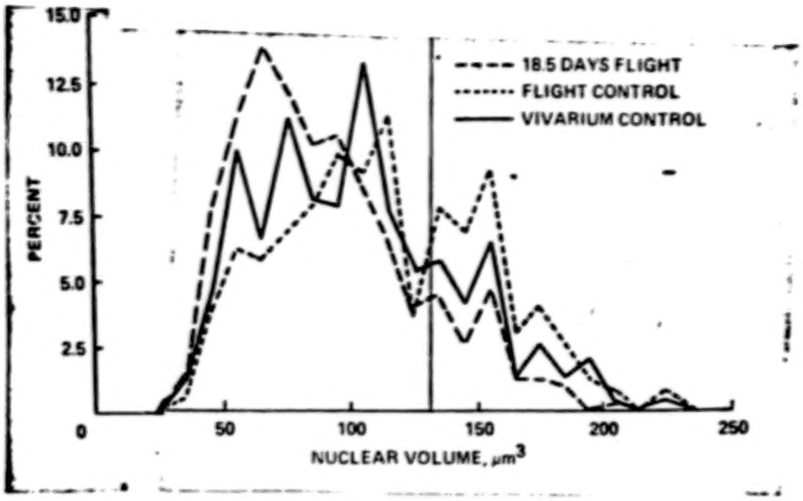
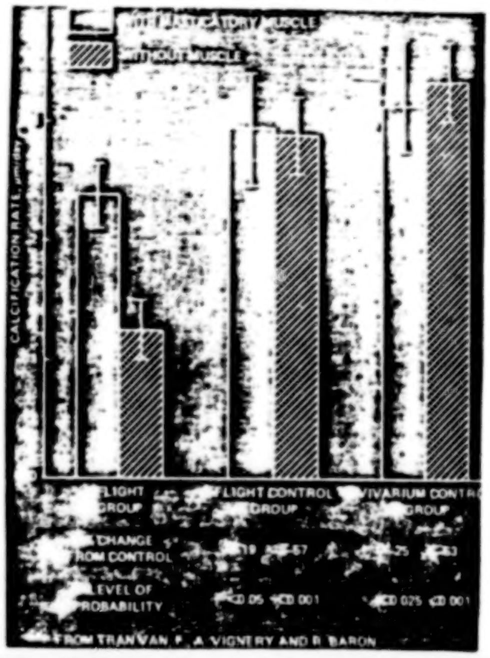
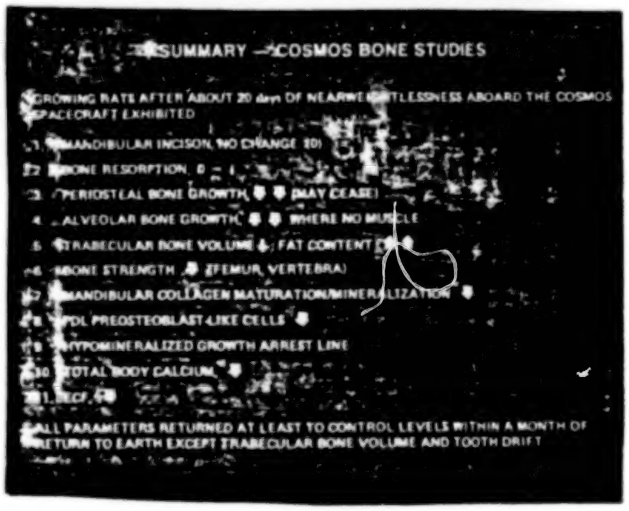


Figure 11.



FROM TRAN VAN P, A VIGNERY AND R BARON

Figure 12.



SUMMARY — COSMOS BONE STUDIES

GROWING RATS AFTER ABOUT 20 days OF NEARWEIGHTLESSNESS ABOARD THE COSMOS SPACECRAFT EXHIBITED

1. MANDIBULAR INCISOR, NO CHANGE (0)
2. BONE RESORPTION, 0 — ↓
3. PERIOSTEAL BONE GROWTH ↓ ↓ (MAY CEASE)
4. ALVEOLAR BONE GROWTH ↓ ↓ WHERE NO MUSCLE
5. TRABECULAR BONE VOLUME ↓; FAT CONTENT [illegible]
6. BONE STRENGTH ↓ (FEMUR, VERTEBRA)
7. MANDIBULAR COLLAGEN MATURATION/MINERALIZATION ↓
8. PDL PREOSTEOBLAST-LIKE CELLS ↓
9. HYPOMINERALIZED GROWTH ARREST LINE
10. TOTAL BODY CALCIUM ↓
11. ECF [illegible]

ALL PARAMETERS RETURNED AT LEAST TO CONTROL LEVELS WITHIN A MONTH OF RETURN TO EARTH EXCEPT TRABECULAR BONE VOLUME AND TOOTH DRIFT

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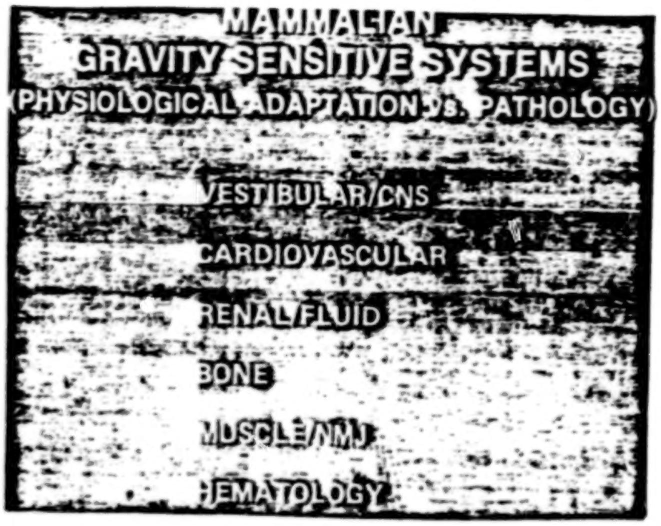
HOLTON TABLES AND FIGURES

Table 7.



MAMMALIAN
GRAVITY SENSITIVE SYSTEMS
(PHYSIOLOGICAL ADAPTATION vs. PATHOLOGY)

VESTIBULAR/CNS

CARDIOVASCULAR

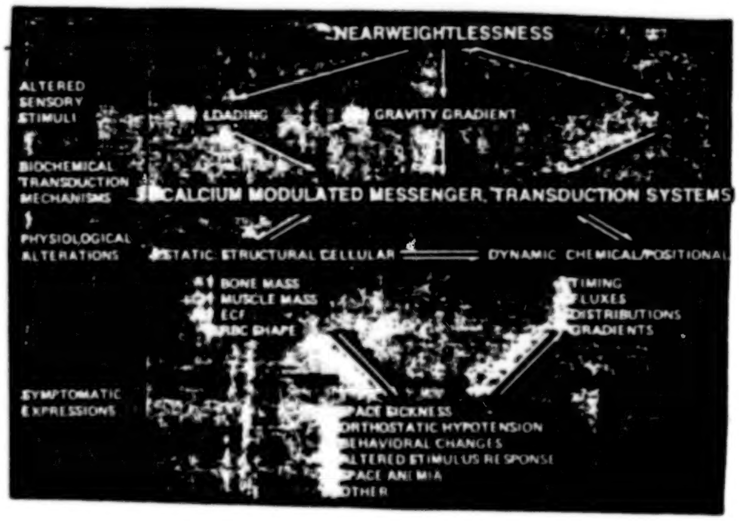
RENAL/FLUID

BONE

MUSCLE/NMJ

HEMATOLOGY

Figure 13.



NEARWEIGHTLESSNESS
ALTERED SENSORY STIMULI
LOADING
GRAVITY GRADIENT
BIOCHEMICAL TRANSDUCTION MECHANISMS
(CALCIUM MODULATED MESSENGER TRANSDUCTION SYSTEMS)
PHYSIOLOGICAL ALTERATIONS
STATIC STRUCTURAL CELLULAR
DYNAMIC CHEMICAL/POSITIONAL
BONE MASS
MUSCLE MASS
ECF
RBC SHAPE
TIMING
FLUXES
DISTRIBUTIONS
GRADIENTS
SYMPTOMATIC EXPRESSIONS
SPACE SICKNESS
ORTHOSTATIC HYPOTENSION
BEHAVIORAL CHANGES
ALTERED STIMULUS RESPONSE
SPACE ANEMIA
OTHER

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DR. ROUX: Thank you very much.

We now have two brief talks which review other aspects of gravity effects on animals, and the first of these will be by John Horowitz from the University of California at Davis, and the title of this talk is gravitational study of the central nervous system.

DR. HOROWITZ: What I would like to do is to discuss selected aspects of information processing by the central nervous system. I will start out by talking about a series of experiments carried out at 1 G, and then I will consider two samples of experiments that were carried out in hypergravic fields, one dealing with the auditory system and one dealing with temperature regulation.

I would like to start with a series of studies at 1 G, to highlight selected aspects of the role of calcium ions in some aspects of information processing. I would like to describe a relatively new technique that has caught the attention of several physiologists over the last few years which is the ability to slice a portion of a mammalian brain and place a thin section of the hippocampus in a chamber. This technique was developed, or at least popularized, by a series of studies in Norway and then at Stanford. A thin section of the brain is still capable of neural activity in a particular neural network. The network has four neurons. With 95 percent oxygen, 5 percent CO_2 over the top of the slice, this thin section can be kept viable for several hours.

The advantage of this preparation is that it is very stable. You don't have to worry about respiratory movement on the part of an animal, and you can see the various cell populations. It is possible to stimulate afferent fibers by placing a stimulating electrode on the slice. One can also insert glass electrodes into the pyramidal cells.

These are relatively large neurons. They are about 40 microns in diameter, so that you can insert a glass pipette within a cell. We have held cells for 37 minutes. Actually individuals have held cells or recorded intracellular potentials for hours and hours, over 24 hours, so that one can have a very stable preparation.

This is a figure of the slice as it appears from the dissecting microscope, and you can see that the layer of cells is visible so that it is possible to take a micro-electrode and advance it and by a change in deflection of the potential recognize that you have a microelectrode inside a single cell and then to place stimulating electrodes, stimulate, and record a potential.

On the basis of experiments like this, certain channels have been identified and one of the channels that has been identified in hippocampal pyramidal cells was by Schwartzcroin. Essentially what they showed was as follows: You can record from a cell that is a hippocampal pyramidal cell in Area CA3. You can pass through the electrode in the cell a slightly depolarizing current so that the voltage shifts and will trigger a burst of action potentials within the same cell. Or you can stimulate the Schafer collaterals, and then you have an action potential that is driven as a result of a postsynaptic response in the pyramidal cell. These records show a series of spikes. They are probably due to the opening of sodium channels and then subsequently potassium channels.

They then block these channels with tetrodetoxin (which blocks vast sodium channels). They have shown that if you depolarize the cell to a much greater extent than previously, by passing a current into the electrode one can open another channel, a channel they postulate lets calcium ions enter the cell.

The channel is voltage sensitive, and they further postulate that this channel may account for a variety of features that are noted in cells, such as a depolarizing after potential, a DAP. The origin of the DAP has been in question, and they postulate that it is this calcium channel that is responsible for the DAP. Therefore, it has been suggested that there is a voltage sensitive channel that has a high threshold, and will open for calcium ions.

There are several other channels that I won't mention, such as calcium mediated potassium channels, but there is one other channel, pyramidal cells that is of interest. These cells have receptors for such neurotransmitters as norepinephrine and dopamine, and dopamine appears to act through calmidulin. At least that has been postulated.

It is a fairly classical idea now that one of the steps at a presynaptic terminal is the entry of calcium ions inside the terminal, and this in some way triggers a series of events that allows a neurotransmitter, in this case norepinephrine to be released from the cell and to the postsynaptic receptors.

An advantage of the hippocampal slice is that you can change the extracellular media surrounding the cells and have a fairly good idea of the composition of the media. So, it is possible to change extracellular calcium and see if you stimulate various fibers what the response will be in the postsynaptic cell. This is a very dynamic system. Norepinephrine is degradated by enzymes such as COMP, MAO. There is an active reuptake for many neurotransmitters, such as norepinephrine or dopamine.

The next series of experiments I will describe were done by Dingledine. This series involved changing the extracellular calcium ion concentration. What they did was apply a constant stimulus to afferent fibers and measure a post-synaptic response. What they found is a change in the calcium-ion concentration from 1.2 millimol to 1 millimolar, to .6 millimolar, the effectiveness of the stimulation drops off remarkably. That is to say a change of .1 millimolar concentration of calcium ion will result in a remarkable drop in the ability to measure a response in the postsynaptic cell a EPSP.

Now I would like to talk about hypergravic fields and a couple of other types of meural processes that, again, involve information processing, but in this set of studies the intent was to look at cell populations as a whole and to determine if there were any changes when the gravitational field was increased. You can increase the gravitational field on an animal by placing it in a centrifuge, in this case a rat.

In the first series of experiments the intent was to see if stimulation of the auditory system was altered in a hypergravic environment, and to do this you can place a crystal on a rat so that you can apply a sound stimulus and record various potentials on the skull of the rat. The voltages correspond to a series of events in the auditory system as a signal ascends the brain stem. The intent is to apply a click that is very precise and very reproducible, even though the animal may shift its head and move about the cage. These experiments were conducted on unanesthetized unrestrained animals.

A control experiment here shows that you do indeed record a series of potentials (Figure 1) as the response to the click is relayed through several nuclei within the brain stem of the rat. This can be shown to be an auditory stimulus by masking with noise.

This series of peaks, an averaged brainstem response (ABR) has been identified by using microelectrodes as being associated with particular nuclei, so that you can name the nuclei which generates that voltage and associate each of the peaks with a different nuclei. I might, also, add that these voltages are remarkably stable. They seem to persist when a lot of other activity is absent. This is a sample of a wave form that is a control. It was recorded from a rat just before it was placed in a hypergravic field. The point I would like to make is that, when the animal is in a hypergravic field there is a shift in peaks.

So here we have an example of one system that is modified by gravity at a gravitational field of 6 G, but it is not modified by gravity at a field of, say, 3 G, and this type of recording in unrestrained, unanesthetized animals allows you to monitor a system. Esperiments show the behavior of a population of cells unlike the earlier experiments where one uses single micro-electrodes and sample a single cell.

I want to now move on to the last topic, temperature regulation and show that it is, also, modified by hypergravic fields. The aspect of temperature regulation I am going to be considering is cold, exposure of animals in hypegravic fields. In this case there are thermistors implanted in the brain and in the core. So, you can instrument a rat like this. One can begin to monitor how well this animal does when faced by gravitational fields.

If you hold an animal for several hours at 1 G there is essentially no change in core temperature. However, even in a field of 2 G, which is a mild gravitational field, there is a fall in core temperature which is quite marked. That is about -2 degrees centigrade.

As you increase the amplitude of the field you increase the fall in core temperature so that by the time you have a field at 4 G there will be really quite a remarkable fall in core temperature. Simply imposing a hypergravic field on a rat will impair its ability to maintain its temperature.

In addition one can cold stress the animal. At 5 degrees centigrade, there is a slight fall in temperature but essentially the central nervous system can activate thermogenesis, so that temperature will remain constant.

In a hypergravic field of increasing intensity there will be a more marked impairment of the ability to maintain temperature. Under the combined stressors of cold and a hypergravic field the rat has an impaired ability to maintain temperature, and this was an unanesthetized rat, unrestrained with no electrodes actually within its central nervous system.

Finally you can begin to dissect this system and ask what parts of the central nervous system are involved and what type of regulation is involved. For example, it is clear here that there is a fall in core temperature, but this could be due to a variety of factors, including suppression of heat production mechanisms, and altered blood flow patterns. There are various mechanisms for heat production, non-shivering thermogenesis (which is a conversion of chemical energy to heat by means of other than shivering).

This is one such experiment that shows this initial fall just at the onset of acceleration is due to a transient increase in flow of blood to the tail of the animal.

Another type of experiment you can perform to dissect out what particular mechanisms of the brain are involved is to measure the oxygen consumption of an animal. These reflect brain mechanisms involved in the control of shivering and non-shivering thermogenesis, and you can see that these mechanisms that would normally be activated in the cold. When cold and a 2 G field are combined these mechanisms are suppressed.

Therefore, in summary, in hypergravic fields it appears clear that areas of the brain stem and hypothalamus are affected differentially. For example, at 3 G the hypothalamus, which is key to temperature regulation is probably modified. However, at 3 G the auditory system is not modified. However, at higher G fields, it becomes clear that the auditory system is imapaired and that signals in the brain stem area are delayed differentially. I would like to comment that the mechanism that underlies these changes in behavior is not known. I wished to contrast the type of experiments that can be performed on individual slices where one can talk about individual channels with the type of experiments that are easily done in hypergravic fields on animals.

I think these experiments on hypergravic fields do show that the brain responds differentially. There is not just a general impairment of function, there is a particular impairment of function that appears to vary from system to system.

The underlying mechanisms for many of these hypergravic studies are, as I said, unknown. It is interesting to speculate that calmodulin may be involved.

Thank you.

DR. ROUX: Thank you.

We have time for a couple of questions.

DR. HOLTON: John, is your temperature effect really a gravity effect or is it simply the fact that you have two stressors superimposed in a synergistic action? In other words, have you ever tried, for example, temperature and immobilization rather than temperature and hyper G?

DR. HOROWITZ: Lowered temperature and immobilization will also, cause a fall in temperature. So, it could be that there are several factors that lead to this observation. However, with the gravitational field there are several observations that are striking. The first one is that this fall in temperature is elicited by a very small increase in the gravitational field. This has also, been observed by Oyama, so that this thermoregulation system is apparently exquisitely sensitive to gravitational changes. It is a relatively new system, evolutionarily speaking.

In regulating the temperature of an animal, you bring into play a lot of systems that were developed in part to serve other functions.

DR. HOLTON: In the Cosmos animals, the data, at least that the Soviets reported showed a decrease in temperature in zero G as well as the other side which makes you wonder if zero G isn't essentially --

DR. HOROWITZ: You sort of wonder if animals have evolved optimally to operate at 1 G.

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Horowitz Figure

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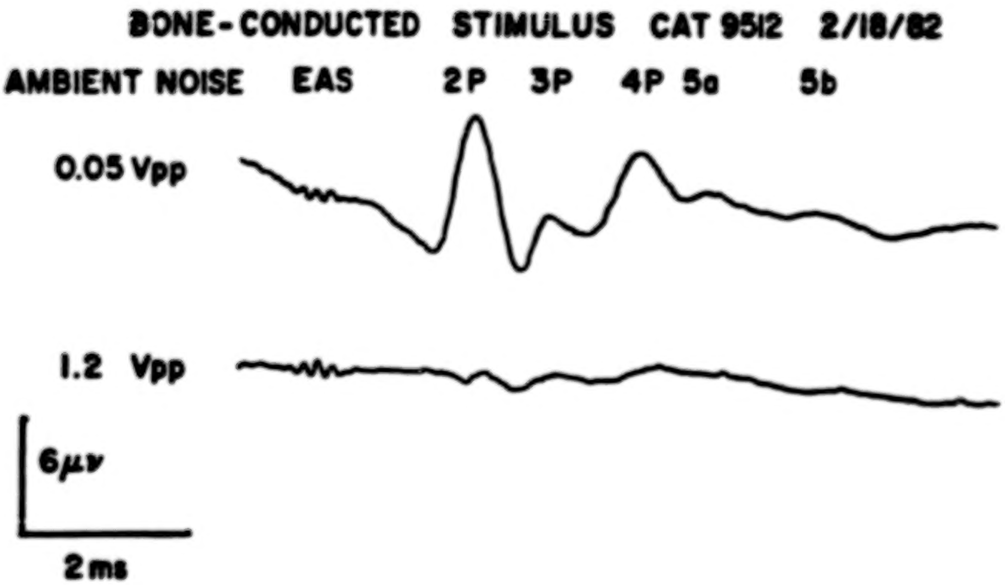

Effects of wide-band noise on the crystal-evoked ABR. Vpp = peak to peak voltage (in volts) of ambient wide-band noise. Each trace represents the average of 1024 samples. EAS = electrical artifact of the stimulus. 2P, 3P, 4P, 5a, 5b = positive peaks of the ABR. Amplitude of the noise was monitored by a microphone placed in the recording chamber. The corresponding increase in intensity is approximately 27 db above the level at .05 Vpp.

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DR. ROUX: Thank you very much.

The final talk of this afternoon's session will be given by George Malacinski from Indiana University, and the title of this talk will be polarity of the amphibian egg.

DR. MALACINSKI: First I will give a brief review concerning the experimental system, and secondly I will deal with a single question today: "Is the activation rotation which responds to gravity, that is adjusts to gravity (I will call it gravity adjustments), a prerequisite for normal development?" The answer to the question is going to be, "no".

That answer is new. It is significant, I believe and it is presently obsoleting a lot of our previous ideas concerning amphibian egg polarity and the mechanism which generates the polarity, and so rather than give a long talk today which would probably involve a lot of double talk, I have decided to focus in on this one question and perhaps in the discussion which follows my talk we can deal with the implications of the answer to this question.

The animal vegetal polarity of the egg appears to be established during oogenesis which makes the amphibian egg a little bit different from, for example, the Fucus egg. That is to say, the construction of the egg during oogenesis results in a deposition of pigment in what we refer to as the animal hemisphere and the heavy or relatively large yolk platelets for the most part, in the lightly pigmented half, which we refer to as the vegetal hemisphere of the egg.

The strict maintenance of the oogenetic polarity, that is to say the pigment in the animal hemisphere versus the large yolk platelets in the vegetal hemisphere has been considered historically to be a prerequisite for normal embryogenesis. As the eggs are shed they exist in a rather random orientation with regard to gravity, such that some eggs are oriented with the pigmented side up, others with the non-pigmented side up, and it has been assumed over the years that the function of the rotation which follows fertilization or activation is to bring all of these eggs into coincidence with gravity such that they all exist after the rotation event, with the animal hemisphere directed upwards or away from gravity.

A histological cross section of the eggs of virtually all amphibians that have been examined, and we have re-examined three in our laboratory, reveal, in fact, that the oogenetic distribution of the yolk platelets, for example, is very rigid. That is to say in the cross sections of these species of eggs one finds the pigment band here which characterizes the so-called "animal" hemisphere, but as we proceed from that animal hemisphere to the vegetable hemisphere we see a gradient of yolk platelets such that the small platelets have always been considered to truly be localized in the so-called "animal" hemisphere and large platelets up here at a quick glance, at least, have been described for the last 50 or 60 years as being accumulated more or less in the so-called vegetal hemisphere of the egg.

Now, as it turns out, however, those correlations with regard to [illegible] are not in fact completely adhered to. That is to say, for the most part small yolk platelets are localized in the animal hemisphere, large yolk platelets in the vegetal hemisphere.

It so happens that the large yolk platelets in general are more dense than the small platelets. We know that because we have taken them out of eggs and placed them on the appropriate gradients. But we have found a rather interesting exception. A small proportion of the platelets in the animal hemisphere, are large and they are heavy, and likewise in the vegetal hemisphere we find a small but significant proportion which are small and are light, and so the oogenetic distribution of yolk platelets first of all doesn't adhere to the simple rule that the stratification from one pole to the other is based upon size and density.

There are exceptions to that rule, and second of all, once the eggs are fertilized and activated we find a violation of the commonly accepted rule, that is to say the orientation of the yolk platelets, vis-a-vis gravity is not strictly adhered to. We find in a rotated egg, one that has been tipped up, some light, small platelets at the vegetal hemisphere.

Considering that the stratification doesn't obey the simple expectation we might digress for a moment and consider what sort of mechanism might be involved in maintaining a distribution of the egg which violates the simple assumption concerning gravity stratification and on the next slide we can see a photograph of the recently published picture (1) from Columbo's laboratory which demonstrates, in fact, that yolk platelets for the most part are surrounded by an actin shell. This is a flourescent micrograph of a yolk platelet which has been stained with a fluorescently tagged antibody, the antibody having been prepared an egg actin, and it appears that the yolk platelets are not really free to move around in the egg cytoplasm, but we speculate by showing the next slide which is probably more of a myth than a fact that indeed, the egg cytoplasm may possess some sort of cyto architecture which involves the linkage of the various sizes of yolk platelets. Here we see small as well as large throughout the egg cytoplasm. There is a priori then, perhaps no simple reason based upon the present data, for expecting that an egg, when it readjusts for gravity orientation would in fact undergo a simple transition of location of the yolk platelets.

The next slide shows activated eggs, and as I have told you, they are all oriented such that the darkly pigmented hemisphere opposes gravity. This is certainly the case for all amphibian eggs that have ever been examined.

Because the eggs do rotate in a uniform way and because in several previous attempts to keep the eggs misoriented that normal development never resulted, it has been assumed that this rotation is, in fact, a prerequisite for normal development, and in fact, as I will get to in a few moments when we approach the last of my slides, postulates have been developed to accommodate shifts of the internal cytoplasm as being prerequisites for normal development. Those shifts actually have been postulated to be the direct result of this rotation of the egg following activation.

Our approach to testing this assumption involved an experiment (Figure 1). The simplicity, I think, is quite dramatic. So went the results. We stripped eggs. I should say by we, my colleague Antoine Neff and myself stripped eggs from a frog and of course they exist in what we refer to as the prefertilization orientation (random with regard to gravity). We fertilized the eggs, and of course they maintain that same orientation if the fertilization is done properly. But then we immobilized them and here we used some tricks which I won't go into now, but the reason this experiment has not been done in the almost century-long history of the amphibian egg polarity problem is that it wasn't possible to immobilize them properly. We immobilized the eggs and just observed their development all the way up through the swimming tadpole stage, and we discovered, in fact, that at least 75 percent of the eggs which are completely inverted survived to the tail-bud stage. The controls of course displays 90 percent or better normal development.

That is to say we conclude that the reorientation of the rotation itself is not in fact a prerequisite of normal development.

The next question and of course perhaps the obvious question concerns the internal cytoplasm of the egg. What we can say right now deals with the so-called cortex, or if you like, the surface of the egg. What we can say from these observations is that there is no prerequisite for events happening in this particular animal or darkly pigmented surface. If we completely reoriented the egg the events which normally would have occurred here actually occurred here. That is to say in normal embryogenesis the small blastomeres form here; the small cells form in the darkly pigmented hemisphere; and the large blastomeres form in the so-called "lightly" pigmented or vegetal hemisphere. In those eggs which we immobilized prior to their rotation, pattern reversal is 100 percent complete. That is to say the region of the egg cytoplasm containing the darkly pigmented cortex, the original animal hemisphere, develops large blastomeres. The reciprocal is true, the lightly pigmented hemisphere develops small blastomeres, and so the development here involves a complete reversal of the pattern.

We can say then that there is nothing irreversibly programmed about the so-called "cortex" or surface of the amphibian egg that at least cannot quickly be changed.

The next obvious question then concerns the egg cytoplasm itself. What is in fact happening as the egg is asked to develop in an upside down condition. We already know that the yolk distribution pattern more or less obeys the simple expectation according to gravity. I have pointed out some exceptions to that expectation, but now we are, in fact, involved in a cytological analysis. All I can say right away is that in fact the shift in the yolk platelet distribution which accompanies the development of upside down eggs is not complete. But I say right now that that is a preliminary observation. That observation is quite complete, however, and that is the preliminary interpretation is that accompanying pattern reversal of upside down eggs a complete redistribution of the small yolk platelets does not result as would be expected to occur if it were a simple gravity reorientation.

Now, the eggs are fertilized after they have been stripped, as they were in the last experiment, but now are permitted to rotate, given a short period of time in the so-called "rotated" condition and then inverted. We are dealing now only with the extreme case that we dealt with in the last slide. Much to our surprise, the development arrests 100 percent at the so-called "gastrulation" stage. That is to say none of the eggs which have had a chance to rotate, to obtain their so-called "normal" orientation after fertilization, and which when put upside down undergo complete development. This was of interest to us. Of course, this result is somewhat in contrast to the previous result. We decided to ask ourselves what is the meaning of developmental arrest? It is a rather gross morphological end point, and we decided, I should say, Hae Moon Chung and myself decided to perform a series of embryological graft experiments to ask the question to what extent has pattern reversal occurred in these eggs which have arrested gastrolation. Although they have arrested and in fact are undergoing death, that doesn't mean a pattern reversal hasn't to one extent or another occurred. We decided to answer that question by using two or three embryological assays. That is to say we took the tissue which normally moves upwards and inwards during the famous process of gastrulation. Those are the so-called "primary inducer" cells. Their activity can be assayed by an embryological graft. Keep in mind these eggs are 1 to 2 millimeters in diameter. We transferred that region to the ventral side, the opposite side of a control egg and determined whether that control egg displayed two sets of axes. That is the so-called "classical" Hans Speyman assay for "primary embryonic inducer" activity.

We examined the inverted eggs which were in the process of undergoing developmental arrest, and we found that indeed the inducer region of those eggs was completely devoid of any pattern reversal, that is to say it did not display any inducer activity.

What we did find when we assayed for the competence of the responding tissue (a rather general embryological concept) to respond to induction was a reversal of competence. We examined that by just grafting that patch of tissue onto various location of recipient embryos and permitting those embryos to go on to develop. These are normal control embryos. This is an inverted donor, and the question was asked, "Is that tissue, when put in various locations, competent to respond to the induction sequence?" If the answer to that question is yes, that means at least partial pattern reversal had been achieved in these inverted eggs.

The answer was, in fact, positive. We are at a favorable location. At our institution we have a genetic colony of amphibians, and we were able actually to do this kind of experiment using pigmentation markers. If you look here you will see the development for example, in this case of an albino recipient which has a patch of dark cells on this side, and the patch is, in fact, derived from a donor embryo which had been inverted. That patch was grafted onto an albino recipient. Here is another case of a dark eye onto an albino pigmentless recipient, that is to say this tissue graft -- obtained from the inverted embryo -- had the competence to respond to induction and development normally.

DR. HEPLER: What kind of animals are these?

DR. MALACINSKI: These are the normal recipients. They are *Xenopus*, although it makes no difference. We have done the experiment with both groups of embryos. In this particular case you are looking at *Xenopus*. It makes no difference.

Now, to the conclusions. To contrast the result from the experiment I just discussed with the first one concerning PreFO eggs is that given sufficient time, that is to say keeping the eggs in the upside down position from the time they are fertilized onward, it appears that the cytoplasmic program can be reversed because those so-called PreFO eggs develop perfectly normally.

If inverted late, however, that is if the eggs are activated and permitted to obtain their normal orientation for a short period of time, 20 minutes or so and then if they are inverted only part of the program of normal embryogenesis is reversed. We hope in the future to exploit this from just a basic embryological point of view. That is, try to determine, in fact, what is the nature of the program which apparently takes time to reverse and reverses at least two aspects of it, one aspect, competence, which we can positively test for and the other, induction, which has been negative so far.

Once activated and once rotated the eggs will go on to cleave for the first time anywhere from an hour and one-half to six hours after activation at room temperature. It all depends upon the species, but prior to the time at which the cleavage fold forms a so-called "gray crescent" or area of pigmentation which marks the future dorsal side of the egg can be recognized. Here is a slide which shows some gray crescents from axolotl, salamander, eggs, and other gray crescents from frog eggs.

The significance of the gray crescent has yet to be completely understood. It serves, however, as a marker for the future dorsal side because in the case of the anuran egg at least sperm penetration actually serves to locate the gray crescent. The gray crescent always forms on the side opposite the sperm penetration point. Anuran eggs are always monospermic; only a single sperm enters, and it is on this side that the cells will make a lop and undergo gastrulation. In urodele eggs it is a somewhat more complex phenomenon; sperm can enter anyplace; more than one spermatozoan can enter an egg, but the gray crescent does reliably indicate the future dorsal side of the egg.

The important point which I wanted to bring up now is the fact that although normally this is the case and normally the dorsal lip forms on the side of the egg which contains the gray crescent, if one rotates the egg for a short period of time, prior to the formation of the gray crescent one can actually program the so-called dorsal lip side of the egg so that it opposes gravity in a rotation in which the eggs are only moved 90 degrees instead of completely inverted. The future dorsal side always develops opposing gravity. That we consider to be quite significant.

Now, the next and last slide deals with the so-called "dorsal-ventral" polarity of the egg, that is, the gray crescent side of the egg and the fact that gastrulation occurs on this side. That is to be differentiated from the so-called "animal-vegetal" polarity.

It has been argued that, the formation of the gray crescent, at least in frog eggs involves a contraction of the cortex towards the sperm entrance point and as the cortex or surface contracts, the yolk cytoplasm becomes redistributed inside of the egg. In fact, we have here a diagramatic representation which goes back several years to some early work by Pasteels. It has been recently resurrected by a group in Holland and a group in Berkeley. It argues that the sperm entrance site causes a contraction of the egg surface toward the sperm enterance site and a redistribution of the yolk such that some of the heavy yolk gets dragged up into the animal hemisphere region of the egg, and it is where it gets dragged up that the dorsal side forms.

I would be freely speculating about the possible role of calcium in programming the dorsal side because the contraction itself may be involved and may require a calcium component. We believe that there is a myosin light chain kinase that is probably active in phosphorylating the light chain of myosin which is a prerequisite for this contraction.

I want to conclude by saying that at the present time, because we have succeeded in getting eggs to contradict the simple expectations of gravity and because we are observing in our cytological sections rather abnormal distributions of yolk which may or may not follow this pattern, we don't really have a good model right now for explaining how eggs are polarized vis-a-vis dorsal-ventral polarity simply because the models to date, including this one, have depended so heavily upon gravity orientation. I am having a change of opinion and moving in the direction that perhaps it is not gravity and as a result, at the present time, we won't discuss how this kind of distribution would come about.

Thank you very much.

DR. ROUX: Questions?

DR. ROBINSON: I was truly startled by your results with the inverted egg, the ones you inverted first or fertilized and had developed and particularly the fact that you found that the small cells were at the top of the egg in what would be the vegetal cytoplasm. Did that indicate a genuine complete reversal of animal vegetal polarity? For example, where does gastrulation begin? Does it begin in the pigment inside now because normally it would begin down in the vegetal side. Where does gastrulation occur?

DR. MALACINSKI: Let us take the salamander eggs which are perhaps the easiest ones to work with at the complete reversal of the site of involution. The involution site forms in a dark region which would normally be small cells but because the dark region is now large cells it turns out involution occurs here, a complete reversal of the site of involution. In the case of Xenopus eggs the location of the dorsal lip is actually a little bit moved towards the margin. It is dramatically shifted. It has certainly moved away from its original lightly pigmented side into the darkly pigmented side but not quite as far in.

DR. ROBINSON: The pattern of these eggs for those of you who may not remember, is that the nervous structure of the Xenopus ends up in the pigmented region. If I take out this neural tissue it is quite pigmented compared to the tissue more ventral to that, and it has long been believed that there is a cytoplasmic pigment that determines where the germ cells are formed. That pigment is in the vegetal hemisphere. So this reversal of polarity is a striking sort of thing.

I mean does the animal develop so that its neural tube is unpigmented and its belly is pigmented?

DR. MALACINSKI: Absolutely, and I did not have time to show you the details of that slide, but on the slide I pointed out the pigment markers are on the bottom because they develop first, but a few of you may have noticed white neural plates on the top and what Ken is arguing is that every embryologist prior to seeing this presentation would have argued, "Well, the neural plate has to be dark black." Well, here are pictures of white neural plates.

DR. KRIKORIAN: How did you achieve this immobilization?

DR. MALACINSKI: It is actually quite simple. It involves shrinking the perivitelline space. The amphibian egg as it is shed contains a vitalline membrane as most animal eggs do which lifts off the surface. It is part of the activation response and it fills with fluid; it is probably inhibition. There is a mucopolysaccharide which is laid onto the top of the egg and water just simply expands it, and now the egg rotates within that membrane. By shrinking the membrane, and there are a number of ways one can shrink that membrane, one then prevents the egg from rotating within these membranes and if you do it right the eggs develop perfectly normally under controlled conditions. It is actually an embarrassingly simple manipulation to do once you know how.

DR. KEEFE: What do you see as your gradient then?

DR. MALACINSKI: I am not ready to speculate right now, maybe in six months, but first of all I am not convinced that there is a gradient per se. I think we generally have tendencies to think in terms of gradients but I would rather not bias the answer and say that I believe that there are gradients.

Ken's argument about cytoplasmic localizations in other animal systems, those yellow crescents and gray crescents that can be followed, well, it may be that redistributions occur. It is just that they don't coincide with the yolk redistribution pattern, and the only redistribution that we can easily see is yolk.

DR. KEEFE: Or pigment.

DR. MALACINSKI: Or pigment, and neither of those reversals is complete, but we are actually involved in a program in which we are sectioning eggs and looking at soluble proteins and trying to determine to what extent they reverse, and hopefully, we will have a complete mixture in a while, and then we will be willing to speculate about whether there are really gradients.

DR. ROUX: Thank you very much.

This concludes our afternoon session. I want to thank all the speakers for really excellent talks, and I am sure those of you who are familiar with the speakers we have on the program tomorrow will agree that we can expect some excellent talks tomorrow as well.

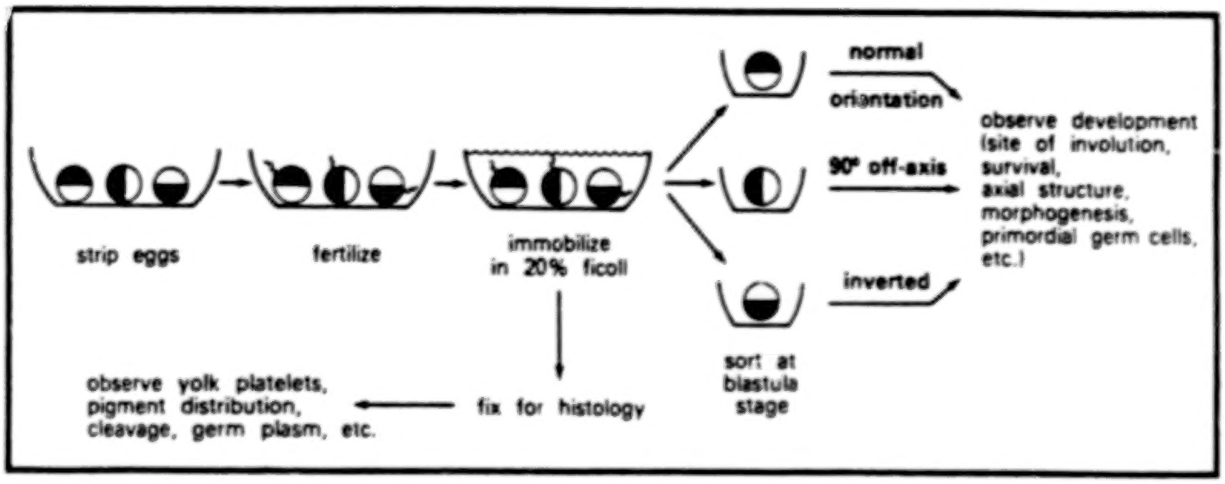
MALACINSKI REFERENCE

Malacinski, *et al*., Differentiation 20:45-51, 1981.

MALACINSKI FIGURE

ORIGINAL PAGE IS
OF POOR QUALITY

design of experimental protocol ⟶ PreFO* eggs (side views)


strip eggs
fertilize
immobilize in 20% ficoll
sort at blastula stage
normal orientation
90° off-axis
inverted
observe development (site of involution, survival, axial structure, morphogenesis, primordial germ cells, etc.)
fix for histology
observe yolk platelets, pigment distribution, cleavage, germ plasm, etc.

*eggs maintained in their "prefertilization orientation" throughout embryogenesis

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DR. ROUX: We have a couple of visitors with us this morning who would like to speak a little bit more with you about the NASA program. This will be especially for the benefit of those of you who are not part of the program right now. I would like Dr. Thora Halstead to introduce them to you.

DR. HALSTEAD: First, I would like to introduce Dr. Gerry Soffen, who is the Director of Life Sciences, the Division of NASA in which space biology is one portion, and life sciences is indeed part of the NASA Space Science Office. He will explain more about life sciences.

DR. SOFFEN: What I want to do first is give you a picture of NASA. Most of you really don't know it. With the exception of maybe Emily and Muriel, who have been intimately involved, you may not realize, first of all, that it is a dynamic agency that is changing. Many of these agencies tend to go on year after year after year, and they are sort of always the same. The grantees come in, the grantees go out, and the agencies stay the same. NASA is not like that. Fundamentally, NASA is not a research organization. That is a very strong thing to say. NASA is an agency whose job it is to exploit space. That is its purpose. There is a thing called the Space Act that was passed years ago that said, NASA, your job, via research or any other technique, is to go and exploit space, whatever that might be.

That is subject to interpretation. As Administration after Administration changes, and director after director changes, the interpretation of that is rather dramatically affected. In fact, in the last couple of Administrations, there was tremendous stress on planetology. There was this tremendous stress on exploring the planets, particularly outer planets. We had Mariners, Surveyors, Vikings, Voyagers that were pushing outward. That has now been, I wouldn't say turned around, but the burner has been turned down. There is much less stress on planetology.

The stress now, with the current Administration -- the attitudes and the way Washington works, with all the advisory boards -- is to push towards astrophysics. Astrophysics is a big thing these days, black holes, quasars, pulsars, space telescopes. That is the rage.

You can see these waves begin to happen as one thing goes in favor, then there is a little less. The one on the horizon is life sciences. There is no question in my mind. Life sciences' day has not come yet. The dawn has yet to come. That is not meant to be a pep talk; that is reality. You will see why in just a moment. It is not here, and all you have to do is look at the budgetary numbers -- and I will say a few words about that -- to realize that this isn't quite the day of life sciences. But it is maybe four or five years in coming. Its day is going to come, and it will be big-time stuff in NASA.

Let me tell you a little bit about the superstructure of NASA. NASA is dominated these days by the space. All you have to do is pick up the newspaper -- and you don't see planets anymore; you don't see rings around Uranus. We are very proud of the Voyager, which is still going. In 1986 they are going to make another pass at Uranus. But the space shuttle is the big thing. The agency is right now in the throes of an enormous decision, on a super-national scale, of what is the role of man in space. Are we are going to go towards robotics and towards automated things, and everything up there will be done by machines, whether it is military or commercial or scientific or otherwise?

That has turned out to be an extremely controversial subject. I remember, perhaps the most telling argument that I heard, of all the people who were talking about commercialization in space and so forth, it was James Michener, who got very interested in space, and, who at a meeting, one of those high-level meetings that one occasionally gets invited to, listened to everybody and said, "I believe that man will be in space, not for any of the practical reasons you say, but because he wants to be there." It is just built into his exploratory nature. For reasons that are intangible, for reasons that you will not get good rationalization for, man is going to want to be there, whether it is this Administration or some other Administration. There are going to be humans in space because he wants to be able to look down on his world, and he wants to physically be there. It isn't enough to have a television camera there or a remote robot device. Man simply wants to be there.

There is another domain that Dr. Halstead has been a pioneer in: trying to find out, what about the biologists? Is there any impelling reason why somebody is going to need space -- not be able to use it -- somebody is going to have an experiment where the only way they can do it is to get out of the gravitational field and do something there? I don't want to develop a bunch of experiments where somebody says, "Yes, I guess I could just as well do my experiment in space," or "let's see what happens when you put something in space."

But there are some experiments, especially when you get at the fundamental biology, the actual stuff I heard yesterday about the way fields are set up and so forth -- somebody is going to have to get away from that big hammer of gravity and actually do their things where there is zero g, essentially gravity-free.

So I see on the horizon just an enormous opportunity for biologists -- not today. Thora asked me, "How am I going to get biologists interested if I don't get the money?" I don't have an answer to that. All I can do is tell you that I am doing my job the best I can; I get all the money I can from NASA, and I split it up the best I can. We are beset with all sorts of problems in the life sciences -- I shouldn't say problems; rich opportunities. We are worried about things like motion sickness, and we are worried about things like origin of life, and we are worried about things like this habitability question. But a domain of that thing is basic biology.

I really want you to hear my deputy. NASA has, as you might guess, like every agency, ways of dealing with things, and different roles that we play. I am the director of life sciences. My job is to run around and make sure that we get a budget and make sure the committees meet and make sure that new investigators are brought in. The day-to-day operation, the actual working of the division, really is my deputy's job. Recently, Dr. Bishop took on about a five- or six-month study -- we were asked the question, what good is a space station? If we are going to have a manned, inhabited station up there, would life sciences want to use it for anything? For the last five or six months, he has been grinding away trying to learn enough physiology and trying to coax a few of your kind of people into writing reports and putting something together. He has actually put a book together. He is going to tell you a little bit about how that happened.

Lastly, I want to finish with Dr. Young. Dr. Young, as I told you, is executive secretary of the American Society for Cell Biology. I am trying very hard to develop some relationships between NASA and other professional organizations, some fraction of his society. Now, I realize that I can be deluged with requests, and there are just so many bucks. But whatever bucks we have, we want to use them as effectively as we can. That is what I really came here to convince you of. NASA is really genuinely interested in your scientific interests. It is open competition. That is the American system. But whatever science we do, we want the absolute best available.

DR. BISHOP: I come not terribly prepared, as Gerry. Let me just tell you, very quickly, so you can get on and talk about science, what is going on about the space station. If you all read the newspapers, you know that NASA is all of a sudden and marvelously interested in something big and new in the sky, and we are calling it a space station, for lack of anything else. That is really not a proper perception, although that is the way the press portrays it. Actually, the space station was the idea back in the late sixties, and the space station is what gave rise to the shuttle that we have just flown this last year. The shuttle was merely the first step towards the space station. Knowing that it couldn't sell something quite as big as the great space station in the sky, NASA backed off and solved the shuttle first, the trucking service to provide the access to the space station. Now it is time to go back and actually go for the space station.

So that got serious this year. The administrator formed a task force, had it reporting directly to his deputy associate administrator, which is sort of the number three person in the agency. That task force is up and running. They have enlisted a number of us within the agency, as well as some contractors, to get some work done. For that task force, we assembled a compendium, very brief, to give to the contractor, saying, essentially, of what use is a space station for life sciences?

It goes beyond that. Let me just say that it is my view, from having done this and from having done some other things that I will mention as well, that the space station can't get along without us. They really can't go ahead and design something for a long-term presence of man in space without the kind of knowledge that our NASA life scientists and our academic community bring to bear about the physiological problems of biology in space. Furthermore, it provides us with an exceptional opportunity to do additional research. I stood up, along with a number of other people and presented to eight contractors, just selected, who were beginning their work of mission analysis, a strong plea that we are exceedingly interested in a space station.

Now, if the space station happens, it will happen at the earliest in 1990, which seems a long way from 1982. But for developing a big piece of technology, it is not. There are a lot of things to do, bureaucratically, in the meantime. We have Phase A studies and we have Phase B studies and we have Phase C studies, and then we start, as the engineers say, cutting tin and start making things.

NASA has gone to eight contractors -- they are the biggies, the Boeings and Lockheeds and Grummans, the people who build really big hardware, largely for the military -- and enticed them to spend a lot of their own money, although NASA gives them a little bit up front, to look at what is the mission for a space station. The mission could include science, and they heard a whole day of science, of which life science was just one piece. It could include commercial opportunities. There are materials processing things already sort of on the drawing boards to do in space, including some bioprocessing things, like electrophoresis to separate cell types and heavy molecules. There are perhaps other opportunities for commercial ventures in space that haven't been explored. There are also, of course, military and national security things that could be done with a space station.

Now, when these contractors say space station, they don't necessarily mean a manned space station. But everybody thinks that that is what is going to come out.

My pitch for life sciences, is that we have one of the early things that gets done on the space station. Obviously, if man is going to be there, one of the first modules is going to be a habitable module. My dream is that the next module will be a laboratory for life sciences, the view being that, for the future, for space station N in the year 2000, what we learn those first few years by doing our kind of work on the space station will directly impact that final configuration.

If the task force is successful, there will be money in the '85 and '86 budgets for the serious design studies. Then, if all goes well, the big money to build the machine, whatever it turns out to be.

There are many ways of contributing ideas. One is to give the ideas to Thora, who is your headquarters advocate for all of those good ideas. The study is being run out of Ames Laboratory. Bill Heinrich at Ames Research Laboratory is the study scientist for this study in life sciences that I described. Dr. Jill Fabricand from the University of Texas Medical Center in Galveston has been our sort of outside chief operative. Give your ideas to any one of those three.

The eight industrial teams will be out canvassing the communities. I suspect they won't canvas very hard in life sciences, because we are not a community that is going to drive the nation to have a space station. It is almost a circular argument for us. We need the space station to do our science, and the space station needs us to do some science at the space station, and so on. It is an internal circular argument. But the other folks, the commercial ventures and national security and such, will need us as part of their architecture.

The companies may be around canvassing. Some of you might hear of somebody coming around to the university saying, what is a space station good for, what would we do on a space station?

DR. MALACINSKI: I would like to ask a general question, perhaps directed to your predecessor, and that is the question of the total pie, the budget pie, for life sciences.

DR. SOFFEN: I will tell you the answer to your question. The more important question is, how do you arrive at that answer.

Briefly, there are six domains. I am happy to give you the numbers, because what I am about to tell you is public information. I get $50 million. Of that $50 million, $20 million goes into a flight program. Forty percent of the money is just to support an ongoing flight program, which has experiments in it. Some things have flown. There is a whole series of flights that we have planned, and most of it goes into pure engineering, like keeping your university heated and so forth.

Of the remaining $30 million, the medical thing that you think about, which is the care, feeding, and health of the astronaut -- pure, simple doctoring of astronauts -- occupies about $5 million. It is called operational medicine, and it makes sure the astronauts go up and come down in one piece.

There is another part that goes into what we call biomedical research. It is some combination of biological research, medical research. It uses animals, it uses humans, and so forth. It is about $10 million. It supports, on the outside world, several hundred investigators.

There is about $5 million that goes into an area called origin of life. We have a very rich program that deals with the biogenesis. We are, in fact, the leading agency for developing the science behind how life was begat.

There is a fourth area that deals with the life support itself. Now, that area also carries a good deal of biology. There is a program called CELSS, which is an attempt to understand how you would go about having a closed ecological life support system. You can imagine the necessity for food that we have there. There is a good deal of biology within CELSS. Some of it is engineering, some of it is not. CELSS is about $4 million.

There is a last area that is pure space biology, unpolluted or uncontaminated with this engineering component. That is about $2 million. So it is, in a sense, the smallest of the segments.

Now, the harder question is, how do you arrive at that? All I can do is give you a one-word answer. By consensus. We have all sorts of people that -- I am ultimately the final arbitrator on that. I have advisory committees, I have subordinates under me, I have people at those NASA centers, I have outside investigators, all that come to bear upon opinions, ideas, thoughts.

Dick, would you just say a few words?

DR. YOUNG: Well, he has given me a golden opportunity to say a word about basic biology in space. I happen to be a strong proponent and one of the pioneers of the field. I think the first egg that was flown in space was probably in 1958, on an old Redstone nose cone at Cape Kennedy. It got about ten feet oft the ground and blew up. And that was the history of early cellular experiments in spaceflight. They didn't work, because we didn't know how to fly.

But over the years, the program evolved. It was always under-funded. It is still under-funded, as you have just heard. But it is a matter of priorities. It is not a simple thing for Gerry to say, well, I am going to put $10 million into space biology, or $5 million or $4 million, or whatever the right number is. What he obviously needs is the best thought-out input from the scientific community that he can possibly get. Are there really good experiments to be flown that make sense from a basic biological point of view? And if so, what are they? Of course, that is what you have been talking about. That is what committees before you have talked about. It, like many others, is an evolving competitive program. It is as competitive as you make it. Its funding picture isn't going to change instantaneously.

The other part of the problem, as I am sure many of you have recognized, is that flying biological experiments in space is difficult. It is extremely difficult. It is the most incompatible environment you would ever want to imagine to do basic research in. Those of you who haven't experienced it, perhaps you will.

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There is potential now. You could, in principle, do your own experiment up there with your own bare hands, if you were foolhardy enough to want to try to do that. Some people will. That day is clearly coming. There is, I think, now real potential for good science in space. It is just starting now.

One other point I wanted to make before I stop is that there is also an international organization that has interest in space biology, and that is the Committee on Space Research, COSPAR, which many of you have interacted with. I happen to be the chairman of COSPAR's Committee on Space Biology. We have biennial meetings. The next one will be in Graz, Austria. We are planning a fairly major symposium on this subject in Graz in May of 1984. You might keep that in mind.

DR. ROUX: Thank you very much.

Okay, we can begin the morning program of the talks. The first speaker this morning will be Muriel Ross from the University of Michigan. The title of her talk is "Calcium Ions, Stores, and Modulators: What is the Gravity Receptor Connection?"

DR. ROSS: There is only one kind of sense organ whose sole normal stimulus would be absent, or virtually absent, in outer space, and that is a pure gravity receptor. So far as I know, in the vertebrate series at least, there is no such end organ. The gravity receptors are sensitive to other forms of linear acceleration, and some of them are sensitive to acoustic vibration as well. Nevertheless, it is just common sense that gravity is a primary stimulus to these receptors, and so we have a very good reason for utilizing the space program in their study.

Another reason, though, that is very compelling is that about half of the astronauts suffer from space motion sickness, which is a variety of motion sickness that some of you perhaps may experience here on earth. This is a very troubling problem, and it is one that we hope to shed some light on.

Currently, the etiology of the disease (motion sickness) is unknown. Some people favor some sort of central phenomenon, and others think that there is a peripheral component (1). So this is another reason to study gravity receptor end organs.

There is still another reason, and that is that the gravity receptors evolved in earth's gravitational field, and no one has the foggiest idea what will happen to them if you keep generation after generation going out in outer space. Will gravity receptors, in fact, degenerate in this near-weightless environment? These are some of the questions that are really important ones and that form a basis for legitimizing our work and our attachment to the space program.

Now, today I am very pleased to be here and tell you about some of the things we have been doing. But I do that on many occasions. What I am more interested in is to have feedback from some of you. My theme song is "Where do we go from here?" I don't know how many of you are entirely familiar with the vestibular system, so we have to start from the beginning. Some of you have heard this before, and I apologize.

We don't have an easy time of studying the inner ear because it is buried in the temporal bone. In some vertebrates, in fact, this bone is exceedingly hard to get into. Even in the human skull, it is the hardest bone. Inside the temporal bone is a structure that is called the osseous labyrinth.

If we take away the bony labyrinth, we will see inside this a membranous labyrinth, which has a very beautiful appearance. Between this membranous labyrinth and the bony labyrinth, there is a fluid which is high in sodium ions and low in potassium ions, a great deal like other physiological fluids. But the series of membranous sacs and tubes is filled with an entirely different fluid. It is called endolymph, and it is high in potassium ions and low in sodium ions. No one quite knows why it has the composition that it does have.

The part of the system that we are primarily interested in here are these two sac-like structures (Figure 1). This is the saccule and this is the utricle. If you look at the drawing carefully, you can just see in outline, the receptor areas. These receptors are commonly called the gravity receptors. The saccule and the utricle are interconnected by a Y-shaped tube, which you can about see here, which leads off into this duct and sac. It is thought that the endolymph is somehow resorbed here in this sac-like ending.

But the point I want to make is this. Even though there is a tube-like interconnection, it appears that the ionic environment of the saccule is kept distinct at all times from the environment that surrounds that receptor area in the utricle. So even though the ionic compositions are quite similar, different things can happen in the two receptor organs, as you will see shortly.

This end, of course, is the cochlea, which is for hearing. Over there we have a series of swellings called ampullae and then the ducts leading off from them. In each ampulla there is a receptor, and that is for angular acceleration.

So we have these two different kinds of receptors (for linear and for angular acceleration) in this complex which is called the vestibular system. This is, of course, the phylogenetically older part of the system.

I want to break my talk up into two parts. In the first part I am going to talk about the crystals that we find in gravity receptors. In the second part I shall talk about the neuroepithelium. But I thought that early on we should just introduce what a gravity receptor might look like, if you were to just draw it, draw what you have seen of a gravity receptor cut in cross-section.

This is a macula (Figure 2). These are called little spots, or maculas. This is the utricular macula, you can tell by its shape. We cut through it and we see a certain arrangement of parts (2). Actually, if we cut through a saccule, we would see essentially the same arrangement. So I will show you only this one diagram.

What we see here is a patch or spot of neuroepithelium which contains the cells which are called hair cells, because they have these thread-like extensions from their surfaces. The hair cells are of two kinds, as we shall see later on in the talk, a type I hair cell and a type II hair cell -- you will see these diagrammed out for you better later on -- and supporting cells. Then over this neuroepithelium, in all the vertebrates, you have some sort of a mass, which is supposed to be there to increase the sensitivity of the underlying receptor area to forces of linear acceleration, including gravity. This mass, in the case of mammals including humans, is made up of a number of small crystals. They are of various sizes, as you see here, but they are small crystals of calcium carbonate in the form of calcite. Calcite is the mineral that is deposited in these crystals in birds and mammals, and in forms below that, you have aragonite, as a rule, but also some vaterite. Of course, in the teleost fishes you have one single mass which is called an otolith. But today I am going to concentrate, really not on some of the comparative things we have done, but rather on the mammalian system.

Now, to give you some idea of the size of the mass that we see here -- you will see also that the crystals are distributed by size along this area so that they are not really all of uniform mass over any particular given area -- but to give you some idea of the size, if you dissected this mass out and looked at it, put it on a stub so you could look at it in a scanning microscope, this whole mass of crystals would be no bigger than the period at the end of a sentence in newsprint. That is how small it is. A pair of the utricular masses weighs something 14-1/2 micrograms and a pair of the saccular masses weighs less than 10 micrograms. So, you see, when we are talking about analytical studies, we face a real problem because of mass.

The crystals are not pure calcite, but they contain organic material, and there is organic material that interconnects the crystals to one another and down to the surface of the receptor area.

If we microdissect patches -- these happen to be rat, but if we looked at human, they would have essentially the same shape -- they look like this. The photographs are simply enlarged. As I have already indicated, the utricular patch is the larger in the case of man and the other mammals.

If you are about 18 years old -- and I don't think anyone in this room would claim to be so young -- you have crystals that look essentially like this (Figure 3). The name given to these crystals is otoconia. Literally, it means "ear dust." These otoconia have rounded bodies, as you see here, and pointed ends. They have faces of a rhombohedron, a rhombohedral crystal of calcite, actually. Here again you can see, I think, the connections, how the organic material actually goes into the crystal.

Now, these are healthy young crystals. What we found rather early in our study was that in the human species the crystals in the saccular end organ begin to demineralize sometime in about the fifth decade of life (3). If you live long enough, in the saccule you will pretty soon have just little patches of end pieces that are left, like this (Figure 4). The utricular crystals essentially remain intact. So this again is some evidence that the milieu around the crystals is not identical for the two end organs.

This meant, of course, that we had a dynamic situation. So we began to explore other kinds of mammals, looking for old animals that would show this kind of a loss. Actually, we haven't found one, and no one else has either. Only man appears to show saccular otoconial loss with age.

We can take any other mammal -- and we have chosen the rat simply because it is rather easy to obtain -- and look at the crystals, and, except for minor differences -- for example, here you see some highly faceted ones -- the crystals all look pretty much the same (Figure 5). They have the same kind of shape. So we really believe that we can take other kinds of species and begin to manipulate systems in order to study these crystals.

One of the things we did, first of all, was to look at the embryology of this system, because we wanted to know how the otoconia formed (4). We saw the central dissolution that occurred in the human saccular crystals. This meant to us that the center had to be softer. The mineral couldn't be as dense in the center as on the periphery. Also we wanted to know if the saccular crystals had something different about them during development to explain why they were so vulnerable.

Let me tell you first off that we didn't see any differences developmentally between crystals at the two sites. In both cases they start out with this threefold symmetry. This material has been decalcified. They form in this fluid-filled endolymphatic space. What was interesting was that you could see, as the crystals developed, that there was a core which then began to accumulate fibrils running in different directions, and forming actually a kind of bow-tie-shaped structure. But definitely there was a center and a peripheral zone.

We then made a great effort to take crystals, embed them in epon, not expose them to any dehydrating fluids or anything. So you are essentially here looking at EM pictures of undemineralized fetal rat otoconia, about the same age as the ones you just saw. You see that now the mineral is more dense in the periphery and very much less dense in the center. If you could compare the two side by side, (demineralized and non-demineralized otoconia), you would see that the pattern of the mineral mimics very much that of the organic material.

Now, when we saw these, a question immediately came to mind. We had been calling the otoconia single crystals. Crystallographers have studied them and have called them single crystals. I was working with a mineralogist. He was insistent that they were single crystals. We looked at our embryological results and asked, can these still be single crystals? And, of course, the answer is yes. It just depends on how these things grow. They will grow together and they will form this perfect crystal, which is what we had been assuming we were looking at.

But I had been in communication with R.J.P. Williams at Oxford, whom some of you may know. I was supposed to go over there and work with him for a while. I suggested one topic, but he said, "No. Why don't you come here and study otoconia? I don't think those things are single crystals." Of course, that was a little bit shocking. But I thought, well, okay, I will go there, because if he doesn't believe they are single crystals, that is a very good place to go and resolve the issue.

We had quite a little bit of trouble in preparing our samples. We broke them up and dispersed them on grids and then studied them on formvar coated grids in the high-resolution microscope. What you are looking at here is a crystal lattice image of Iceland spar. The spacings that you are looking at are only about three angstroms apart, something like that. What you are really seeing are the reflections of the calcium atoms. If you look at this -- this is a lattice image -- you see the periodicity goes over this whole fragment. You really don't see anything except distortions that you can account for. But this is a very pure crystal of calcite.

Now, when we look at the crystals, broken fragments, of any of the specimens that we were dealing with, you do not find that. You find instead domains. Actually, this one little fragment in here does have the lattice image on it. I am sorry it isn't high enough magnification that you can see. We did the electron beam diffraction work. This is the pattern that was obtained from this fragment, and it looks pretty good. It looks like it should be a good calcite crystal. There are imperfections, however, because there are extra dots. The dots that are in very regular arrangement indicate a nice crystal. But the extra dots indicate that there are imperfections in these crystals, and we found that in every case.

Moreover, what we found, rather surprisingly, was that the calcite of the mammals mimicked the organization of aragonite. We never expected that

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at all, because calcite is the most stable form. Nevertheless, the calcite in mammals seems to mimic the aragonite that evolved through the reptiles.

This is one of the rat otoconial fragments (Figure 6), and you will note that it has one pointed side here. But it has a curved edge. We don't have a lattice image on this because we couldn't tip this crystal over far enough to see it. We had a limited range on the goniometer stage. But if you look at the lines, they are perfectly arrayed. We are convinced that this is one of the smallest crystalline units you can obtain out of otoconia. But it meant that indeed the otoconia were not single crystals of calcite. R.G.P. Williams was right. We have since been able to section the adult otoconia without decalcifying them. This is just embedded in epon. We find many more of these rounded-appearing fragments.

Now, you might say, what difference does it make? Well, it makes a lot of difference, because single crystals of calcite are symmetrical crystals and they would not be piezoelectric. But the first thing that comes to mind when you see crystals that are arranged as these are -- sheets with organic material between them -- is that they could be piezoelectric.

Moreover, the crystals aren't perfect, the little individual crystals are not perfect. They have these rounded edges and so on, and so they can, in fact, be piezoelectric. This is an important point, and I will come to that in just a moment.

SPEAKER: Could you explain what piezoelectric is?

DR. ROSS: Piezoelectricity means that if you take certain classes of crystals and put a stress on them, they will produce electricity or they will become polarized. The other thing is if you put these same crystals in an electric field, they will become deformed. They will change sign as the stress changes. They become polarized in a strict relationship to the amount of stress that is put on them, and you can reverse the polarity or the sign.

I think some of you already realize how important that point could be in comparing a gravity situation with a weightless environment. But I will mention more about it in just a moment.

The one thing I wanted to say is that we tried to attack the question of the composition of the organic material. We had to use microdisc gel electrophoresis (5). This gives you some idea of the size of the gels we were dealing with. One exciting thing that came out of that is that we did get a peptide or a protein that was about 17,000 molecular weight. I know that calmodulin has always been described as an intracellular substance, and yet we are excited about this. This may be some sort of calcium-binding material. We are presently pursuing this with HPLC methodology. So we hope that maybe in a couple of years we will have this thing worked out.

We think that one of the more important things to do right now is to resolve the question of the organic composition, because this is what is collecting the calcite and putting the carbonate together with it, making the little crystals, protecting them, and keeping them intact through most of our lives.

Moreover, not only could otoconia be piezoelectric, but they are supported or suspended from organic material, a glycoprotein, that could be piezoelectric itself. The otoconia and the otoconial membrane are situated in an environment in which there is an electric field, because we know that if you measure the field from endolymph out to perilymph in the other end organs -- no one has done this in the gravity receptor, so far as I know -- there is about a 120-mV drop. So if we put a piezoelectric system in here, we can actually modulate the strength of that field and the effect down on the hair cells.

There is, of course, a catch in that; we would also have to have the electric effect directed properly toward these hair cells.

Now, I became really interested in this because there is a big question whether or not the kinocilium which is present -- one kinocilium and several stereocilia are arranged in order, as you see here is motile. It has all the internal ultrastructure of a motile kind of cilium. It has a basal body and it has striated rootlets, as you will see. So I really believe that it is motile and that what we are doing with the electric field is modulating kinociliary activity with the stereocilia following.

I began to model this (Figure 7). I can't go into this much today. I don't have time. But I began to model this on bioenergetic principles. I am coming to believe that mechanical, electrical, and chemical energies are all interchangeable in the system (6).

Now, I wanted to spend the rest of my time on these striated organelles. I had a visit from Leonard Cipriano in connection with some space shuttle work, and he said, "What if someday they can fix material on the shuttle, in a space lab? Would you be able to do that?" We talked about the fact that you couldn't use osmium, and might have to collect animals on day three and leave them for five days before you could do anything more with them on the ground. You couldn't use formaldehyde. I said, "Well, Leonard, I don't know, because we have always fixed things in Karnovsky's fixative, which includes formaldehyde, and we have had such good luck with it. But we will try, we will try to develop something."

So we beheaded some animals. We just very quickly opened up the inner ear part in fixative that was just three percent glutaraldehyde in phosphate buffer. We fixed them for two hours at room temperature, and then we put them in the refrigerator and we left them there for five days. No one in their right mind working on the ear has really ever done this. Then, after five days, we went ahead, pretended now we would be on the ground, and we post-fixed them using one percent osmium in phosphate buffer. We like phosphate buffer, fortunately. We had the tissue embedded and so on.

Carol, my research associate, went down and looked at the tissue first, and she came back up and she said, "I don't think it is fixed well. Most of the mitochondria look okay, but some don't." I had seen some mircrographs, and I said, "Well, the tissue didn't really look that bad." We never throw anything away in this lab; we always look at everything and as we decided to look further at this tissue.

This is one of the first things we saw. This is a striated organelle that had never been described before, under the upper end of the calyx. Now, I want to back up. This type I hair cell is completely surrounded by a nerve ending called the calyx. The type II hair cell doesn't have a calyx. It just has an afferent bouton ending down here, and these are efferents, which maybe I will have a chance to talk about. I don't think so, though.

Anyway, here was this large striated organella. I said, "What is that?" Carol said, 'Well, it looks like a rhizoplast." I said, "Fine. What is a rhizoplast?" She had studied botanical things, and she said, "It is like a fibrous rootlet."

So this is an entirely serendipitous finding. When we went back to the literature and began to look, we had accidentally used exactly the fixative recommended to preserve rhizoplasts. It was just by chance. But here we have these striated materials that, to me, look a lot like muscle.

The other thing is that all the striated elements in the hair cells were beautifully preserved. We began to see the rootlets of the kinocilia that people were having trouble seeing, and so on and so forth. I just want to show you some of these striated elements.

I am interested in this proving whether or not the striated elements are contractile. This is where I am hoping some of you can interact with me later on. We have repeated our experiment. We have treated one side with Karnovsky's and the other side with this glutaraldehyde fixative. We know that the only way we see all the striated organelles is with our new fixative. Some of you who know about microtubules will notice we have microtubular arrays showing up as well.

First of all, I would like to show you (Figure 8) a striated rootlet on a kinocilium in a supporting cell. So far as I know, no one has really dealt with it as a single rootlet coming down from the center of the basal body. But we are convinced that there are at least two rootlets here on the kinocilium from a hair cell. The one drawback with this fixative is that it always disrupts the cell. Other people have reported this, no matter what their fixation in the inner ear. But we don't really see that with Karnovsky's, although we are seeing it with glutaraldehyde. We are trying to solve this problem by increasing the tonicity of our fluids.

Here is a little nubbin of another rootlet. We have seen this so many times that we are convinced that there are at least two rootlets on hair cell knocilia, but there may be more. The other interesting thing is that we see the basal body connections to dense material that is at the edge of the cuticular plate. We have better pictures of this. Why I say there may be more than two rootlets is that I have seen a rootlet like this in position right up against the cuticular plate.

So I think we really still don't know how many striated rootlets there are on hair cell kinocilia. They are very thin. You need to cut them within ten degrees of their axial arrangement in order to see them at all. So it is just by luck that you do find them.

But the interesting thing is, they are arranged in the direction in which it is currently thought that movement of the stereocilia will cause depolarization of the cell. That is, movement of the stereocilia toward the kinocilium depolarizes the cell, and movement in the opposite direction hyperpolarizes it. So we know that at least one pair of these things is in the plane of movement if the kinocilium is motile.

Here you see some striations at the edge of the cuticular plate, which are showing up all the time. If you cut the cuticilan plate at an angle you see the dense stripes and the fine line in between with the filaments. It has been a big source of discussion over several years now, whether the cuticular plate is striated or not. You say, what difference does that make? Well, again we are talking about a possibly contractile mechanism that becomes involved during the transductory process. This is an exceedingly important issue, and it is one that we hope, again, that you will be able to give any insights on.

This is a saccular type I hair cell showing exactly the same thing. We found type I hair cells at the crest of the ampullar end organs also have these striated organelles. The type I hair cells in the vestibular system have them. The type II hair cells do not have them. There is not a

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comparable organelle like this in the hair cells in the organ of Corti. But you do find some striations in the cuticular plate.

Here I want to emphasize that this organelle is suspended from electron dense material right at the periphery at the zonula adherens, and the cuticular plate is also suspended from that.

So what you have is a mechanism, really, I think, for bringing signals in from the surface to the zonula adherens, which is below the occluding juction. Then in the type I hair cell you have a mechanism for bringing the stimulus in under the calyx. Here you can see some of this material that comes down under the calyx, the upper end of the calyx, and I think you can see the striated nature of it. Now, this material is at the opposite end in the calyx from ribbon synapses. They would be down at the base of the cell.

We don't know what the studied organelle does. Again, this is something that those of you who are interested in calcium and so on can perhaps interact on.

I want to emphasize a couple of things here (Figure 9). One is that, although it has been demonstrated that there is actin in the cuticular plate, you see that the microtubules are very closely related to it. In looking in the literature, I have found only a couple of people who have talked about this kind of relationship. At higher magnifications, you definitely can see some of the filaments that are coming right down onto these tubules. Of course, because both these arrays probably are existing in a low-calcium medium and are under calcium regulation, you have a mechanism in the type I hair cell for telling the lower end of the cell what is going on at the upper end, through the microtubular arrays.

If anybody knows what these granular structures are from their plant studies, please tell me. I looked for an hour-and-a-half in the Journal of Cell Biology, just flipping pages to see if I could see anything like this anywhere. Again, Carol brought in a paper that was written by her mentor. He worked on rhizoplasts in plants. He had these same granular structures, but did not identify them. So far that is the only place I have seen them. So if you know what they are, I would really like to know. One guess might be ribosomes with glycogen or something, but for what reason I don't know.

The stereociliary rootlets, by our method of study, are not uniform in appearance. They are definitely also striated. This has really some significance for me when I am thinking about the evolution of this system.

I will come back to the idea that what we have here is a system in which we could have an electrical field modulation affecting the interaction of the kinocilium with the stereocilia and a mechanism through the striated organelles for bringing this information across the occluding junction and down to the hair cell membrane below that, so that we can affect the electrical activity of the membrane in this way. I think that is one of the most important conclusions that you can reach.

The other thing is, of course, that the type I hair cell has this striated organelle that is lacking elsewhere. So we have something else going on. To me, again, this might be important in interacting with you, because the type I hair cell is very sensitive to certain drugs, such as streptomycin. The type II hair cell is not. Again, I hope that maybe our findings will provide some clue as to why this is true.

Finally, because I can't go into the evolution of this, I was very excited to find out that invertebrates have mixtures of kinocilia and microvilli on their surfaces that can act in concerted action. Then in a recent abstract I read that came out of Peter Satir's laboratory (7), an epithelial cell was described that had microvilli and kinocilia intermingled; both were calcium-sensitive and both responded in integrated fashion to calcium. So calcium is going to figure prominently in hair cell activity and I hope that in the workshops and elsewhere you can give me some insights.

DR. ROUX: Any questions?

DR. BANDURSKI: You made the observation that mechanical and electrical and chemical energies might be interchangeable. In the mid-1950s there was developed an inertial navigational device called a Solion. This thing depended for its activity -- if you move any charged particle in a fixed voltage, you can in fact generate an amplifiable current. I think that would be the exact analogy from the physical world with what you spoke of here. The Solion, I don't think, ever really developed very far, for other reasons. But it is a beautiful thing. It could detect changes in acceleration, it could detect light, it could detect pressure, and, of course, in every case you are simply moving a charged particle in a fixed voltage.

DR. KEEFE: I am struck that there is the potential here for running a physical course from an otoconium right down through the otolithic membrane, through the membranes of the stereocilia and acting across those, through the rootlets, on down through the cytoskeletal system, in which one does not have to imply a chemical transduction mechanism, strictly a physical mechanism. I am wondering whether or not in that system the two hair cells aren't different.

DR. ROSS: Oh, yes, I think they are, and this is where I need some input. Microtubules in one and endoplasmic reticulum in the other. Yesterday we heard about how the two are interrelated in the mitotic spindle. But here they are apart. We do see some smooth endoplasmic reticulum.

I want to add one more thing that I forgot, because I think it is important. I think we are beginning to come up with some evidence that there is a peripheral component to motion sickness. That, to me, is a most exciting thing. That is why I really feel we need the input from you experts.

DR. LEOPOLD: How do you mean, a peripheral component?

DR. ROSS: Well, we have tried to prove whether or not these crystals are piezoelectric. We have a problem with a very small mass. Maybe someone here can give me input on that. But if they are piezoelectric, they are under stress already in a gravitational field. Just that one thing, taking them out into a near-weightless environment, would change the stress on the crystals and would alter their basic background electric activity against which inertail force would then act. But aside from that, if we are talking about these striated organelles, I am beginning to have all kinds of ideas about how these things might be working in a normal cell. Then you begin to think about what would happen in this near-weightless environment.

DR. MALACINSKI: How are the crystals connected? You see these threads, but are the threads continuous with the crystals?

DR. ROSS: Yes. If you cut through them -- other people have done this -- the material goes right into the inside.

DR. MALACINSKI: So they may be the same composition as the crystals.

DR. ROSS: They may be. We don't know. That is what we hope we are going to solve.

DR. LEOPOLD: But you thought that was protein, didn't you?

DR. ROSS: Oh, it is. It is glycoprotein. I have done a PAS stain. I know it is glycoprotein.

DR. BENNETT: I think we should take a comparative approach to this idea of piezoelectricity and transduction. Hair cells elsewhere in the vestibular system work very well without any crystals. As Hudspeth has shown, the hair cells work very well in the frog sac when you take the crystals away and you poke at it. It is exquisitely sensitive.

DR. ROSS: If I may interrupt, Hudspeth works in a very artificial medium which is high in sodium and low in potassium. This is a fluid similar to what Flock has used, and Flock has consistently called attention to the fact that this is an artificial medium. So it is true, Hudspeth has taken away the kinocilium and the basal body and has said the kinocilium was unnecessary. I think if you look at this, you can see that that would be true, because it really doesn't matter if the kinocilium is present if you stimulate the stereocilia under artificial conditions the striated organelles are still there.

I think that Hudspeth's recent work is that there is an extracellular current that he can detect that goes down the stereocilium from the tip to the base, when he stimulates them, micromanipulates them, in an *in vitro* situation. I have no doubt that he can do this, and he does very elegant work. But I think that he also says, then, that these are like electrically conducting cable and that the current just flows through the cell and out the bottom end. I think there is no way that you really can come to that conclusion looking from these.

But I agree with you; one has to look. If I had been able to do the evolutionary thing, the interesting thing is that the frog has no type I hair cells, as we know them in the mammalian system. There are lots of other things that I would be glad to talk to you about.

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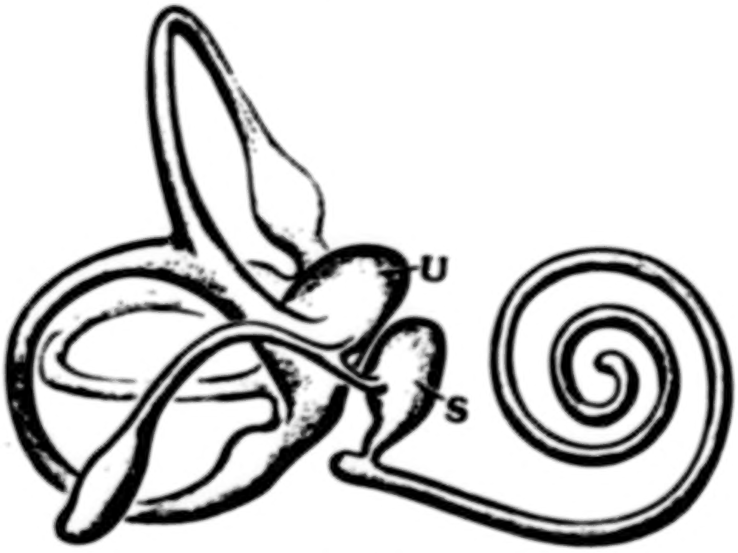

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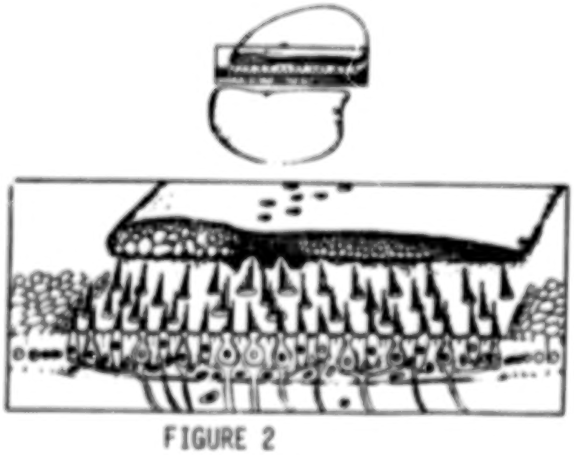

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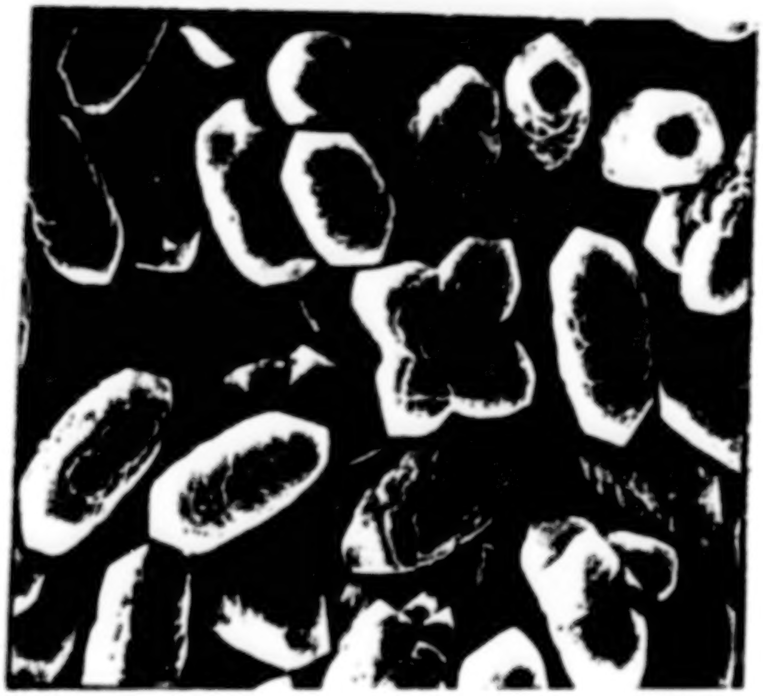

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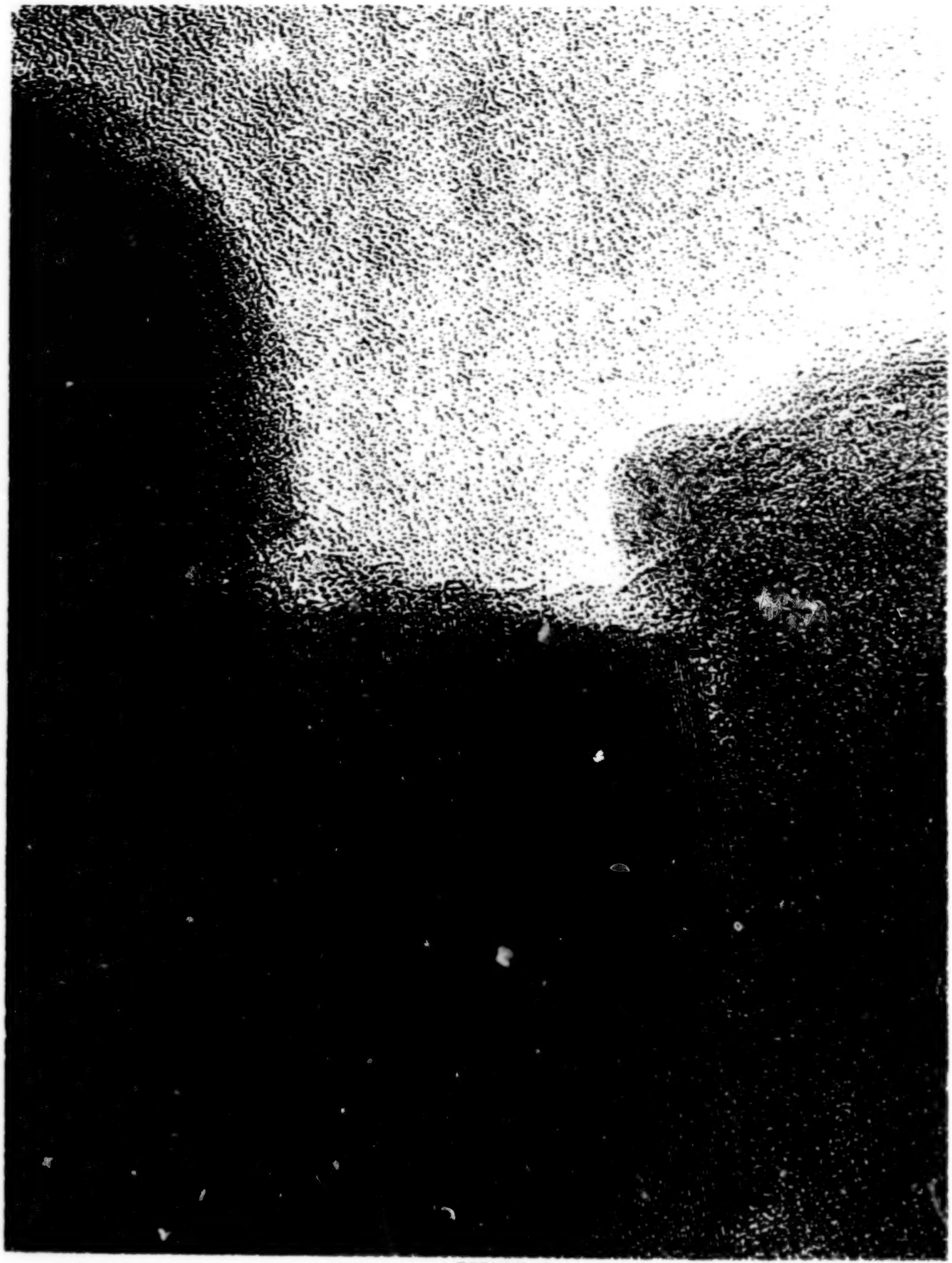

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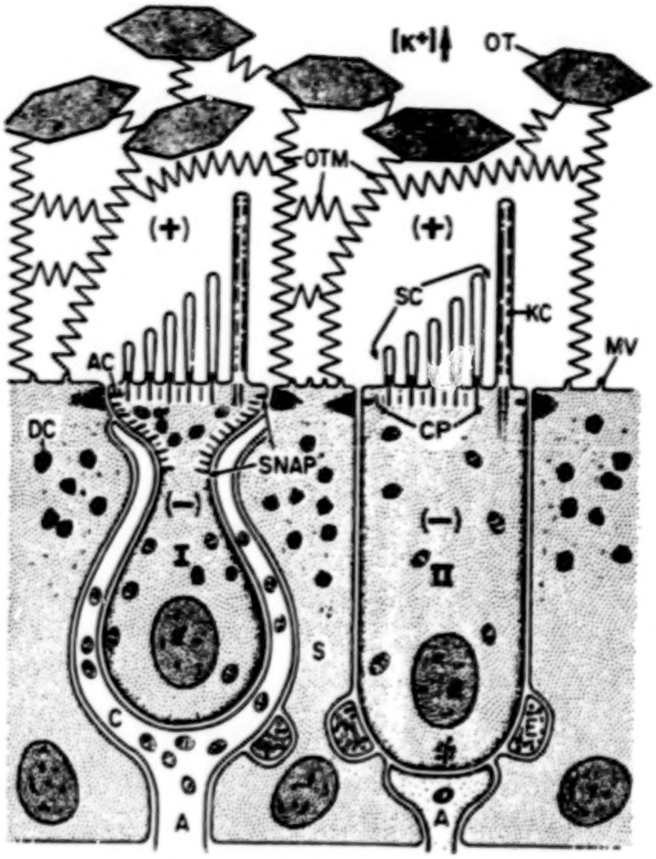
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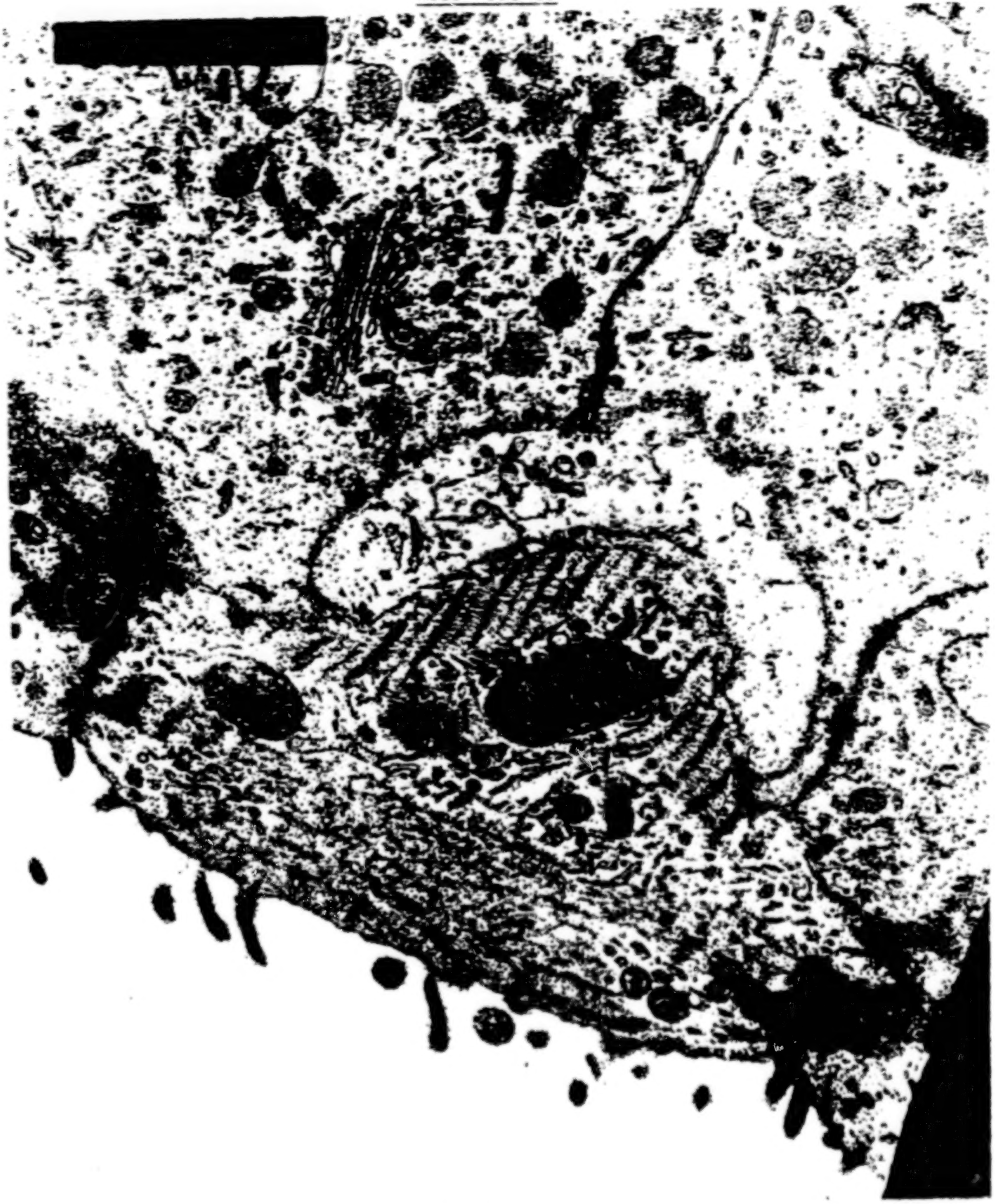

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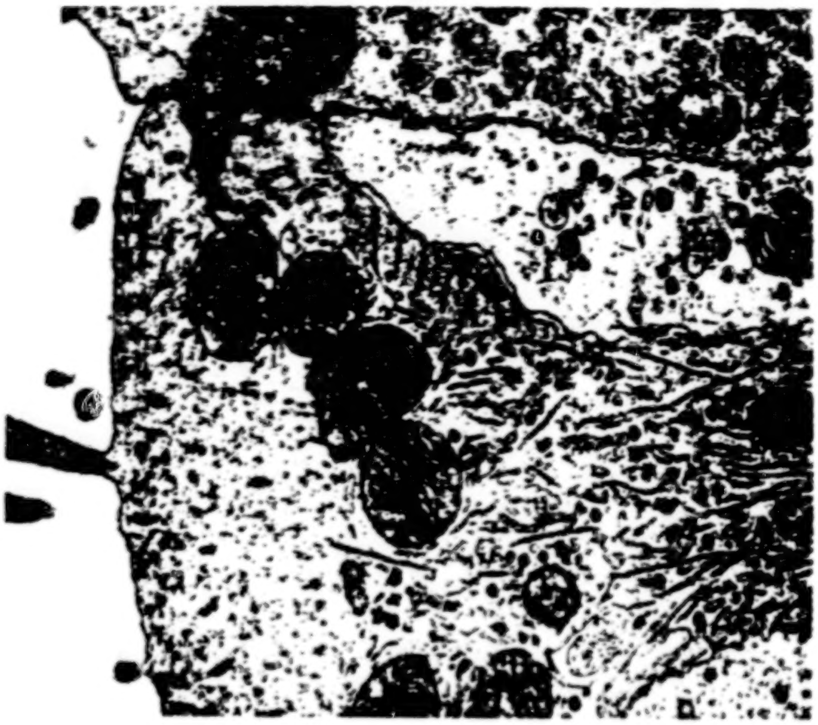

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DR. ROUX: I think we have to move on to the next talk. As you can tell, we have split our schedule a little bit. We will have the break after this next talk. The next talk will be given by Dr. Howard Rasmussen from Yale University. The title of his talk is "The Plasma Membrane Calcium Pump."

DR. RASMUSSEN: I am going to try to cover three aspects of cellular calcium metabolism in animal cells. The first, is the importance of the plasma membrane in calcium homeostasis. The second, a recent group of experiments dealing with the actual mechanism of the calcium pump. The third, is consideration of the function of the pump in relationship to the mitochondria and to the function of calmodulin in the intact cell.

Figure 1 is a schematic representation of what we currently think we know about cellular calcium metabolism in an animal cell. The single most significant fact is that the external calcium concentration is about 1,000 micromolar, whereas the free calcium ion concentration in the cytosol is somewhere in the range of 0.1 to 0.2 micromolar. There is a 10,000-fold gradient of calcium across the plasma membrane of every animal cell.

The other membrane that is illustrated is the inner mitochondrial membrane. The other compartment, which is of general importance to cellular calcium metabolism, is the mitochondrial compartment. Its membrane is, as you know, energized, and there is an active calcium pump in it. Until recently, we really didn't know what the ionic concentration of calcium was in the matrix space, but recent data suggest that intramitochondrial calcium ion concentration is not much higher than that in the cytosol.

Inside the mitochondria there is also a large pool of nonionic calcium. If you want to reason teleologically, the major regulator of cellular calcium metabolism over the long run is the plasma membrane. What is remarkable is that our heart cells, for example, have the same calcium concentration today that they did five years ago. So there is some way that this cell -- even though in the heart cell, for example, every time there is a systole there is an entry of some calcium into the cell -- is able to precisely regulate its calcium content. This is critically important, because it turns out that this mitochondrial pool of calcium can only expand to a certain amount. If you damage the cell membrane and increase the permeability of that membrane to calcium, this system in the mitochondria is the second line of defense of the cell against calicum intoxication. If you exceed the capacity of the mitochondria to store calcium, the cell dies because of the activation of phospholipases and proteases and an uncoupling of oxidative phosphorylation.

The remarkable thing about the plasma membrane is that, in spite of this calcium ion gradient, the cell only expends about five percent of its basal metabolism in maintaining this gradient. The reasons are: first of all, the plasma membrane is relatively impermeable to calcium under normal circumstances. So the rate of entry of calcium into the cell is low. Second, there is a calcium pump in the plasma membrane which drives calcium out of the cell against its concentration gradient.

Figure 2 is a schematic representation of the kinds of pathways that we think operate in the plasma membranes of many animal cells. From left to right, in some cells that are excitable, whenever the sodium channel opens, some calcium enters the cell through that mechanism. In a variety of other cells there are two regulated channels called, respectively, potential operated channels -- which says that when the plasma membrane potential falls, the channel opens and calcium enters the cell -- and so-called receptor operated channels, in which the interaction of a hormone or a neurotransmitter with its receptor leads to the opening of a channel without necessarily a change in membrane potential. Then there are two energy-linked mechanisms for removing calcium. One is calcium ATPase, which we are going to talk about, and the other a sodium-calcium exchange process, in which extracellular sodium exchanges for intracellular calcium. The sodium gradient that is established by the sodium potassium ATPase is the driving force for moving calcium out of the cell via this pathway. I am not going to talk about this pathway, although it is an important mechanism of calcium efflux from a number of cells.

Now, many animal cells, when they are stimulated by neurotransmitters or hormones, demonstrate two phenomena. These are seen, for example, in isolated cells from the exocrine pancreas. These cells secrete digestive enzymes in response to acetylcholine. If you load these cells with calcium 45, so you have labeled intra- as well as extracellular-calcium pools, and then add an analog of acetylcholine, there is an initial release of calcium from the cells simultaneous with an increase in the calcium ion concentration in their cytosol. The precise location of the pool of calcium that is released isn't clear, but it is probably the plasma membrane.

Then, over time, there is a net accumulation of calcium by the cell. This is a reflection of a sustained increase in calcium influx into the cell across the plasma membrane. So during a two hour period, the cells show a net total accumulation of calcium. When you then add atropine, which is a blocker of the receptor, and the initial pool refills, i.e., it gains calcium, and then the cell pumps out the accumulated calcium from the intracellular pools.

But different cells have different patterns. For example, in the smooth muscle cells, a way to judge how much of the calcium for contraction of that smooth muscle is coming from outside the cell and how much is coming from a pool that is released from the plasma membrane or sarcoplasmic reticulum is to examine cellular response in the presence or absence of extracellular calcium. When the response of three different branches of the mesenteric artery to norepinephrine are examined in the presence or absence of external calcium, the cells don't all respond the same. Removal of calcium blocks the response of some arterioles completely, but others show almost half the response that they showed in the presence of external calcium. Hence different cells show different patterns of calcium release and calcium uptake during initial cell activation. But in all of them, calcium uptake is a common event and wherever a sustained response is observed, there is a sustained increase in entry of calcium into the cell. This fact appears rather paradoxical if what I told you is true, that calcium is a cellular toxin.

In the next part of this talk studies concerned with the mechanism of action of the plasma membrane calcium pump, will be discussed. The technique which we have used is to make inside-out membrane vesicles from human red blood cells. You take human red blood cells and you lyse them under the proper conditions and treat them in a low ionic environment, and their membranes vesiculate with what was the inside of the cell, now outside. So in fact they have resealed. They are intact osmotically. But the surface that is outside was the cytoplasmic surface of the original membrane. This is obviously an ideal object for study because now you can manipulate the internal calcium concentration, ATP concentration, magnesium, and so on.

If you take intact red cells and load them with calcium 45 when they are depleted of ATP, and when there is ATP present in the cell, the rate of calcium efflux is different. It is clear that there is an ATP-driven efflux of calcium out of the red blood cell, and that is what we are going to study in the inside-out vesicles from human red blood cells.

The rate or extent of calcium 45 accumulation into the inside of these vesicles is measured as a function of time. That is equivalent to moving calcium out of the cell. You can look at it in two ways. You can measure the response in terms of ATP hydrolysis, or you can measure it in terms of calcium uptake. In these membranes there is a very low level of magnesium-dependent ATPase in the absence of calcium. ATPase activity is stimulated slightly on the addition of calcium. But if, in addition to calcium, you add the calcium receptor protein calmodulin, there is a marked enhancement of calcium ATPase activity and calcium uptake.

If you take these membrane vesicles and make your incubation medium contain only an anion that isn't permeable to the red cell membrane, or is sparingly permeable, then the rate of calcium uptake, even in the presence of calmodulin, is very low. If you add two millimolar phosphate, the rate of calcium uptake increases dramatically.

Now, for the plant physiologists, what you need to know is that there is an anion channel in the red blood cell called band 3, which is a protein that exchanges phosphate for chloride for hydroxyl for bicarbonate, and is very important to the function of the red cell.

So the presumption is that when you add calcium there is an accumulation of calcium and, as you will see in a minute, the development of a membrane potential.

If you have no permeant anion that goes through band 3, then the rate of calcium accumulation is slow. As soon as you add an anion like phosphate, it is driven electrophoretically, if you will, into the vesicle, collapses the membrane potential, and calcium uptake is markedly enhanced.

SPEAKER: Were these studies done with calmodulin?

DR. RASMUSSEN: Yes, these are all with calmodulin.

Now, the way you can prove what is going on is to treat the red cell, before you make these vesicles, with a substance called DIDS (which is an abbreviation for a long name) which specifically blocks the anion channel. It prevents the movement of phosphate or sulfate through the red cell membrane. Our results show that calcium uptake in the presence of phosphate, ATP, and calmodulin in normal vesicles is high, but in DIDS-treated vesicles in the same medium the uptake rate is low. So by blocking the anion channel, you block phosphate uptake and the stimulation of calcium uptake by phosphate.

I just have to show you that with several different methods for measuring membrane potential, in the absence of phosphate -- in other words, in the presence of an impermeant anion, if you have calcium, calmodulin, and everything present and then add ATP to start the reaction, and have ANS or $DiSC_3$, both of which are potential sensitive dyes, you develop a positive membrane potential. You can measure this potential in another way by using tracer amounts of 35_S. The prediction is, if you have developed a positive potential, positive inside the vesicle, and you add tracer amounts of 35_S, you would get an uptake of 35_S, and the ratio between the external and internal concentrations of 35_S gives you a measure of membrane potential.

It comes out, in the presence of calmodulin, that you develop a potential of about 70 millivolts.

There are two possible models which might account for all of the observations I have told you (Figure 3). One is that the calcium pump is electrogenic. In this model (Figure 3-right), the ATP would drive calcium in. The accumulation of calcium would create a positive internal potential and drive anions through band 3. The other possibility is that the pump operates as a calcium-hydrogen exchanger (Figure 3-left). When Ca^{2+} goes in, H^+ comes out, and then you develop a hydrogen ion diffusion potential, positive in. This potential would then stimulate anion uptake. If you treat the cells with DIDS and make the vesicles, you block anion uptake. The prediction is that with either model, you would decrease calcium uptake (Figure 3). So those experiments don't allow you to determine which of the two models is correct.

SPEAKER: Why does the exchange model there give you --

DR. RASMUSSEN: This model? Because you have a hydrogen ion concentration gradient and a hydrogen ion diffusion potential.

SPEAKER: It doesn't change.

DR. RASMUSSEN: Yes. Hydrogen ion is high outside and low inside, and you develop a hydrogen ion diffusion potential which gives you the inside positive, and that would drive anion uptake.

Now, there are two ways to get anions across membranes, other than through an anion channel like band 3. There are certain substances, like acetate buffers, in which there is some small amount of nondissociated acid (HA). Acetate will cross the membrane in its undissociated form (HA). If you take DIDS-treated vesicles in the presence of acetate buffers, you can make a prediction of what should happen. The prediction is that if there is a primary electrogenic calcium pump, then in the presence of the acetate, there will be little stimulation of calcium uptake. What would happen is that the acetate would come in, dissociate, and you would soon develop a hydrogen ion gradient in place of a calcium gradient. So you would not have electrical neutrality. Hence acetate would not stimulate, according to this model (Figure 4). If you had acetate and added FCCP, which is an ionophore that makes a proton channel in the membrane, you would predict FCCP would have little stimulating effect if the pump were an electrogenic one.

On the other hand, if it is a calcium-hydrogen exchange mechanism (Figure 4), you would predict that the addition of acetate would stimulate calcium uptake, because this uncharged species (HA) would enter the vesicle, hydrogen would exchange for calcium, and the anion would neutralize the charge on calcium. You would predict that under these circumstances the addition of FCCP would have little effect. So this experiment allows you to determine which of the two models is more likely to be correct.

The other kinds of anions are certain lipophilic anions like nitrate and thiocyanate, which go through the membrane essentially as the charged species (Figure 5). So in this case you would predict that the addition of nitrate would lead, if the pump were an electrogenic calcium pump, to a marked increase in calcium uptake. On the other hand, the addition of this kind of anion in a system operating as a $Ca^{2+} \rightleftharpoons 2H^+$ exchanger would not stimulate calcium uptake, but if you now added the ionophore FCCP and made a proton channel, so you now delivered both anion and hydrogen ion into the vesicle, you would stimulate the process (Figure 5).

Figure 6 summarizes an enormous amount of work, but shows you that in the presence of undissociated weak acid, acetate, in a DIDS-treated vesicle, calcium uptake is stimulated. Likewise, in the case of nitrate, calium uptake is low in the presence of nitrate and no FCCP, but uptake is high in the presence of nitrate and FCCP (Figure 6).

So let's go back. What we found was that acetate enhanced calcium uptake and that it wasn't influenced very much by FCCP. In fact it was suppressed slightly, about 10 to 15 percent. So the acetate data is consistent with a model of $Ca^{2+} = 2H^{+}$ exchange (Figure 4). Also when we added the lipophilic anion nitrate, in the absence of FCCP, there was no uptake. There was about five percent stimulation over the control. That again makes the electrogenic model very unlikely. When we added FCCP as well as the anion, we got a marked uptake of calcium (Figure 5). So both sets of experiments are consistent with the notion that the plasma membrane pump operates as a calcium-hydrogen exchanger.

Now, in the red cell this exchange doesn't lead to any change in internal or external pH, because of these anion shunt pathways through band 3. So you can essentially get the equivalent of a hydroxyl = chloride =bicarbonate, whatever, kind of exchange you want. So there is no change in pH. But if you had a cell in which the anion transport properties were different than the red cell, then the potential exists that when you activate the calcium pump, you actually lower the intracellular pH.

Now let's go back and talk about calcium metabolism. We are shifting from the red cell to other cells. But so far, in every animal cell in which it has been looked for, this pump is present in the plasma membrane and is regulated by calmodulin. I believe, on theoretical grounds, you can predict it will be found in every animal cell.

This is a picture again of calcium metabolism (Fiugre 1), 1,000 micromolar in the ECF, and, 0.1 to 0.2 in the cytosol. As we study more and more animal cells and develop better techniques for preparing freshly isolated mitochondria from freshly isolated tissue, the actual amount of exchangeable calcium in mitochondria goes down. There is not a lot of it in smooth muscle cell mitochondria when that muscle is intact. What happens is that when we prepare animal cells with collagenase or something else, and they go through a long series of incubations, and then we make the mitochondria, there is an enormous amount of calcium in the mitochondria. So in fact all the studies that are done with isolated hepatocytes and other types of isolated cells deal with cells that have already taken up more than their normal amount of calcium into the mitochondria.

What I want to do is think about calcium metabolism in a liver cell, in terms of the behavior of the plasma membrane pump and the mitochondrial membrane (Figure 7). The data in this figure shows the rates of calcium efflux, out of the cytosolic compartment of the cell by way of either the plasma membrane (left) or mitochondrial membrane pump, as a function of cytosolic calcium ion concentration.

The key point is that when the cell is at rest, the cytosolic calcium is near 0.1uM. When it is activated the cytosolic calcium increases. What this slide says is that when you increase it fivefold from, say, 0.1 to 0.5 micromolar, the important membrane that protects the cells is the plasma membrane. So when you just tickle the cell with a hormone, and there is a very small rise in cytosolic calcium, there is practically no increase in calcium in the mitochondria, and practically all the calcium that goes in gets pumped back out of the cell, with a delay (because it takes a little while for the calmodulin to activate the pump). However, if the cell is maximally stimulated and, if you go up to 1 micromolar Ca^{2+} in the cytosol, the mitochondrial uptake of calcium begins to dominate.

If this conclusion is right, it has two important implications. First of all, it says that in the steady state it is very unlikely that any cell operates for very long above 1 micromolar calcium in its cytosol. The reason is, if the mitochondria in our liver cells operate in the cell the way they do in isolation — and there is some indirect data that suggest they do -- at one micromolar free calcium, it would take only two-and-a-half hours for these mitochondria to become overloaded. The cell can't operate at that level of calcium when it is turned on for any period of time.

So in fact, if this idea is correct, the data predict that in a turned-on cell, operating for a long time in response to a hormone, it is likely that the calcium ion concentration in the cytosol is going to be somewhere here; circa 0.4 - 0.6uM. So, in a sense, what I am saying is that the control range over which the calmodulin-regulated enzymes, or any other calcium-dependent enzyme, must operate is from 0.1 to 1.0uM, but when activated for a prolonged period it probably operates around 0.5uM.

With this conclusion as background, it is now possible to consider how calmodulin, the calcium receptor protein operates. In all of the calmodulin-regulated systems in which it has been critically looked at, calmodulin turns enzymes on only when there are three or four calciums bound to calmodulin. In other words, you can't have a calmodulin-response element complex with one calcium associated with the calmodulin. However, there is more to the story than simply binding. The data in Figure 8 show the calcium-binding curve for calmodulin. If calmodulin operated in the cell the way it does in isolation, the data would predict that in order to turn a calmodulin-regulated process on, the cytosolic $[Ca^{2+}]$ would go from 0.1 or 0.2 micromolar to 100 micromolar. If what I just told you about cellular calcium metabolism is correct, the cell can't possibly operate at 10 let alone 100uM.

DR. WATTERSON: What enzyme activity is that?

DR. RASMUSSEN: This is just calcium binding to calmodulin.

However, if you take the same amount of calmodulin and add a small amount of phosphodiesterase -- phosphodiesterase is a calcium-calmodulin-activated enzyme -- and you measure its activity as a function of $[Ca^{2+}]$, you see that at about 0.1 to 0.2 micromolar there is very little activity, and by the time you have reached 1 micromolar, it is fully activated. I have chosen conditions to show it exactly that way. This is similar to the kind of range I just told you about from other considerations that must operate in a cell.

This is cheating a little bit, because, depending on the ratio of calmodulin to phosphodiesterase, you can move this curve in a parallel fashion. The more calmodulin you have in relation to a fixed amount of phosphodiesterase, the further over to the left this curve will be. These concentrations happen to be the ratios that are found in a liver cell. In brain cells in fact the ratio is a little bit higher. The qustion of interest is how one explains these quite different curves (Figure 8).

Wang and Huang, Blumenthal and Stull, and Klee have all done some very elegant kinetic measurements, which I am not going to go through but from which, if you make a few simplifying assumptions, you can come out with a model that looks something like that depicted in Figure 9. The interactions between calcium, calmodulin, and a response element system is ordered and highly cooperative. The most important change is the following, if you consider it as a three-step association reaction: the first step is calmodulin binding cooperatively to calcium, so once the first calcium goes on, the second automatically goes on. That brings about a conformational change in calmodulin that allows it to associate (step 2) with phosphodiesterase (PDE) with an affinity about 100-fold greater than the form of calmodulin with no calcium. This association of PDE with Ca_2CaM leads to a conformational change in calmodulin that allows the next two calciums to associate with a 1,000 to 10,000-fold greater affinity (step 3) than to the free calmodulin.

Only when three or four calciums are bound to calmodulin does the phosphodiesterase go from the nonactive, or relatively nonactive, to the active form. This is not only true for phosphodiesterase, it is true for the calcium pump in the plasma membrane which is also activated by calmodulin. This seems rather paradoxical, if you are using calcium as a message, that calmodulin, the receptor for the calcium message, actually regulates the calcium pump in the plasma membrane, because it appears to be turning the calcium message off as soon as it gets started.

In those cells in which a hormone has a sustained effect, there is a continued influx of calcium: i.e., in cells like kidney cells that are treated with parathyroid hormone, influx remains high as long as the hormone is present. What happens is that calcium efflux is activated by the calcium-calmodulin mechanism. Its rate almost matches that of influx, thereby minimizing the net accumulation of calcium by the cell. In my view, the activation of the calcium pump by calmodulin is a critical component of this control system which minimizes calcium accumulation by cells while allowing the cell to utilize calcium as a message.

In Figure 10 I have tried to indicate what I think happens during the time when a hormone acts on a cell. When you have a resting cell (Figure 10-top), the calcium ion concentration in the cytosol is low. The calcium ion concentration in the mitochondrial matrix space may be one-and-a-half to two times greater than that, but no more. There is a small amount of calcium stored in an exchangeable but nonionic form in the mitochondria.

When you activate the cell, and upon initial activation with the release of that bolus of calcium and the influx of calcium, at a time when the calcium pump is not activated by calmodulin, there is a transient rise of calcium in the cytosol, somewhere in the range of one to a few micromolar (Figure 10-middle). When that happens, at 1uM calcium, there is an uptake of calcium into the mitochondria. This expands the exchangeable calcium pools in the mitochondria. So, in fact, you need much more calcium than you would think to turn the cell on initially because of this mitochondrial uptake.

When you activate the plasma membrane calcium pump via calmodulin, the steady state calcium ion concentration in the cytosol falls, the rate of calcium uptake into the mitochondria falls, and you reach an new equilibrium (Figure 10-bottom). At equilibrium, or steady state, the calcium ion concentration is going to be in the range of 0.5 micromolar, and the major efflux pathway is the plasma membrane.

DR. ROUX: Any questions?

DR. BIKLE: Howard, a quick question on the positive cooperativeness and calcium binding. I think you implied in Figure 8, but you didn't say it, that if you were to take *in vitro* an isolated preparation of calmodulin and phosphodiesterase, you could show a positively cooperative binding of calcium to calmodulin that you didn't get in the absence of phosphodiesterase. Is that experimental data?

DR. RASMUSSEN: No. That comes out of the kinetic data. The problem is, all of those studies are done with a huge excess of calmodulin, so you have a 100-fold greater calmodulin than you have phosphodiesterase. A very small percentage of the calmodulin you are studying is actually going through that series of association reactions.

DR. BIKLE: Can't you design the experiment to --

DR. RASMUSSEN: Well, it is hard to do because, in fact, one of the ways the cell gets the requisite sensitivity is to have the calmodulin concentration much higher than the phosphodiesterase concentration. It is theoretically possible to do the experiment, but it hasn't been done. Again, calmodulin is small compared to the response elements. So you have to have a specific way to look at a conformational change in calmodulin. When it is free, you can see that. When the first calcium binds, the second goes on cooperatively. But when it is associated with phosphodiesterase, you can't see it.

DR. BIKLE: You can't see calcium binding to phosphodiesterase-calmodulin components?

DR. RASMUSSEN: Well, it is very difficult.

DR. ROBINSON: I didn't quite understand your argument about the electroneutrality of the calcium, and how you were suggesting that the membrane potential is then being generated. You are saying that the hydrogen diffusion potential builds up inside the cell. So you are saying that there then is a hydrogen gradient under those circumstances.

DR. RASMUSSEN: Yes, in the original vesicle, with or without DIDS, with a nonpermeant anion, you see a membrane potential. If you add phosphate, the membrane potential collapses.

DR. ROBINSON: You are suggesting that that membrane potential is a hydrogen ion diffusion potential.

DR. RASMUSSEN: Right. That was possibility one, and the other was that it was an electrogenic pump. But the experiments we did seemed to indicate that it is the former.

DR. ROBINSON: Well, I don't quite understand, then, if the pump is neutral, why it doesn't work perfectly well against a large membrane potential.

DR. RASMUSSEN: There is no source of hydrogen ion inside.

DR. ROBINSON: So it stops working not because it builds up a membrane potential, but because it depletes the hydrogen ion inside that is necessary for the exchange.

DR. RASMUSSEN: That is sort of a semantic kind of argument. In other words, any way that we supplied it with hydrogen ion, it worked, whether we gave it an acetate or we gave it nitrate and FCCP and increased hydrogen permeability to the membrane.

The one experiment we can't do, you see, is, when these inside out vesicles are made, you have to make them in very low ionic media, so you can't have a large buffer pool in the vesicle. If what I am saying is right and I had a hydrogen ion buffer system of 10 to 15 millimolar inside there, I would have essentially a source of hydrogen ion, and then, presumably, I could get a higher and sustained rate of calcium uptake than I see. I can't do that experiment because I can't make inside out vesicles under those conditions. So that is a limitation of trying to do that.

DR. BIKLE: Well the other explanation is that you don't know whether this is electroneutral or not. If you have a one-to-one exchange with protons and calcium -- and there is evidence for that, for example, in mitochondria -- then you are going to build up the potential.

DR. RASMUSSEN: Yes, but if that were the case, the potential would be due in part to a calcium potential.

DR. BIKLE: Right.

DR. RASMUSSEN: And then, according to the experiments we did, we should have gotten intermediate results in all of those kinds of experiments.

DR. BIKLE: I don't think so.

DR. RASMUSSEN: Yes.

DR. ROUX: One more question.

DR. CLELAND: Is there a sufficient pH gradient built up in these vesicles to be measured with a pH probe?

DR. RASMUSSEN: I think so. We are doing those experiments now. Tentatively, yes.

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RASMUSSEN FIGURES

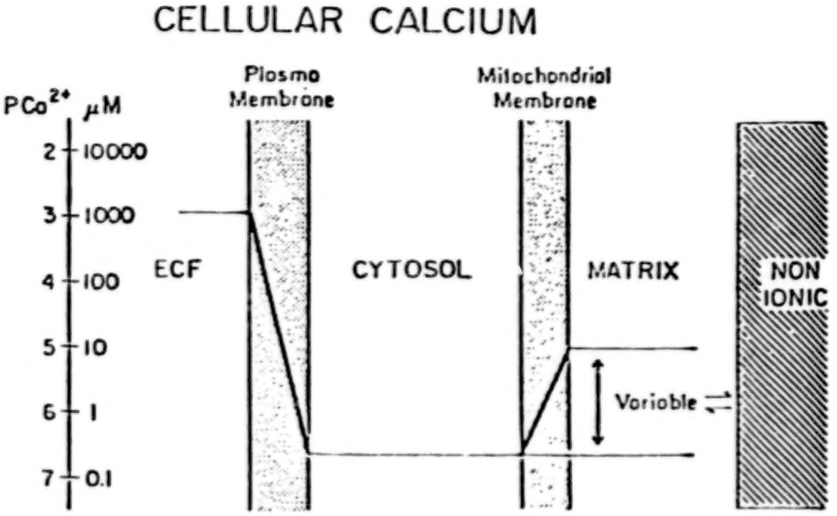
CELLULAR CALCIUM
PCa^{2+}
μM
2
10000
3
1000
4
100
5
10
6
1
7
0.1
Plasma Membrane
Mitochondrial Membrane
ECF
CYTOSOL
MATRIX
NON IONIC
Variable

Figure 1. A schematic representation of cellular calcium metabolism. The extracellular $[Ca^{2+}]$ is 1000 μM, the cytosol ion concentration is 0.1-0.2 and that in the mitochondrial matrix space between 0.2 and 2 μM. There is an exchangeable, non-ionic pool of calcium in the mitochondrial matrix space (right) in equilibrium with the ionized calcium in this space.

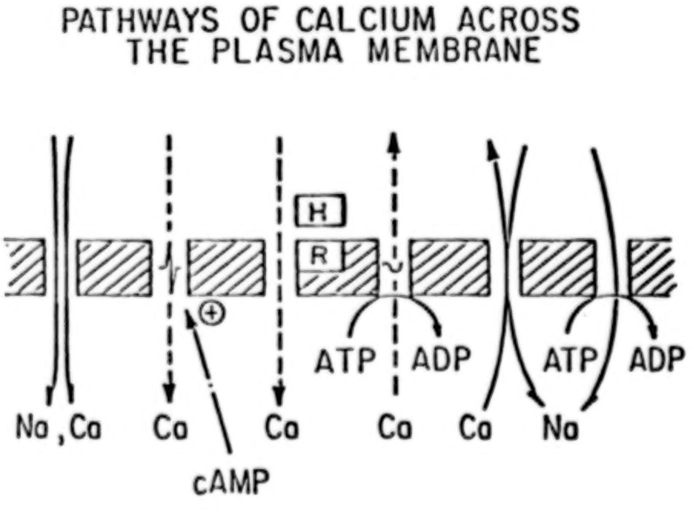
PATHWAYS OF CALCIUM ACROSS THE PLASMA MEMBRANE
H
R
⊕
ATP
ADP
ATP
ADP
Na, Ca
Ca
Ca
Ca
Ca
Na
cAMP

Figure 2. The pathways of calcium influx and efflux across the plasma membrane.

C-3

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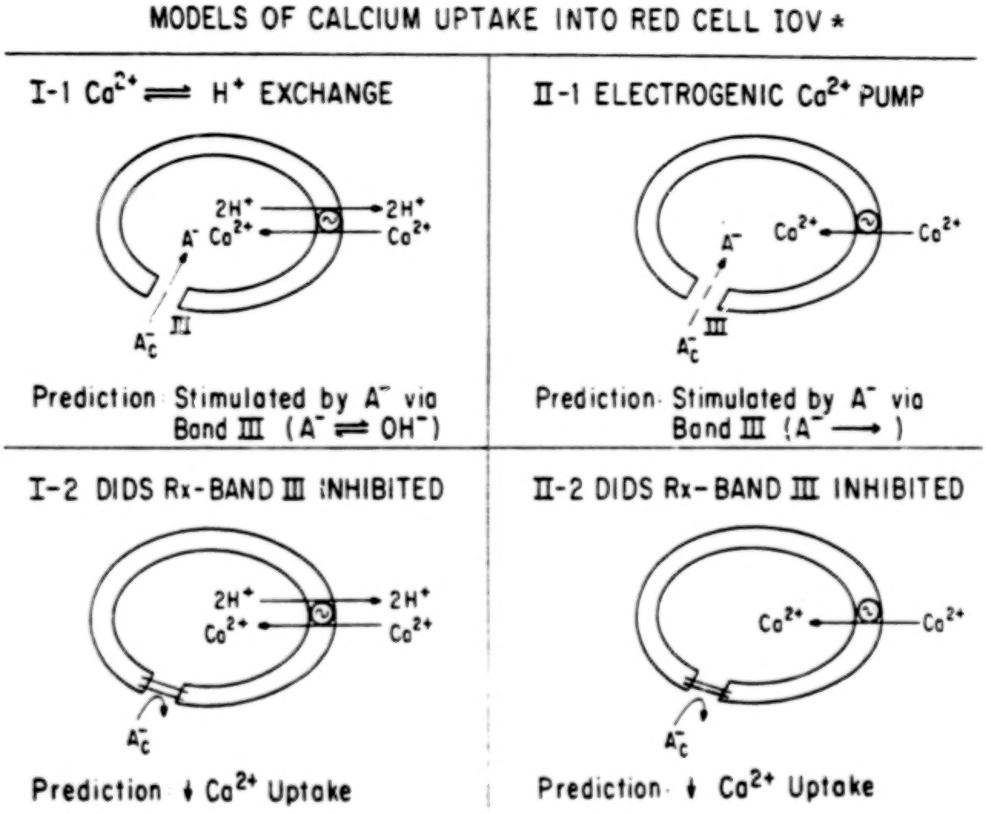
MODELS OF CALCIUM UPTAKE INTO RED CELL IOV *
I-1 Ca²⁺ ⇌ H⁺ EXCHANGE
2H⁺
2H⁺
A⁻ Ca²⁺
Ca²⁺
III
A⁻c
Prediction: Stimulated by A⁻ via Band III (A⁻ ⇌ OH⁻)
II-1 ELECTROGENIC Ca²⁺ PUMP
A⁻
Ca²⁺
Ca²⁺
III
A⁻c
Prediction: Stimulated by A⁻ via Band III (A⁻ ⟶)
I-2 DIDS Rx-BAND III INHIBITED
2H⁺
2H⁺
Ca²⁺
Ca²⁺
A⁻c
Prediction ↓ Ca²⁺ Uptake
II-2 DIDS Rx-BAND III INHIBITED
Ca²⁺
Ca²⁺
A⁻c
Prediction ↓ Ca²⁺ Uptake

Figure 3. Two alternative models by which the plasma membrane calcium pump operates: left, Ca^{2+}-H^+ exchange model; right, electrogenic calcium pump. III represents band 3, an anion channel in the membrane which carries a variety of anions ($A_{\bar{c}}$). DIDS is a specific inhibitor which blocks band III and inhibits anion-stimulated calcium uptake regardless of which model operates.

* IOV = inside-out vesicles. These models are shown in Figures 3-5.

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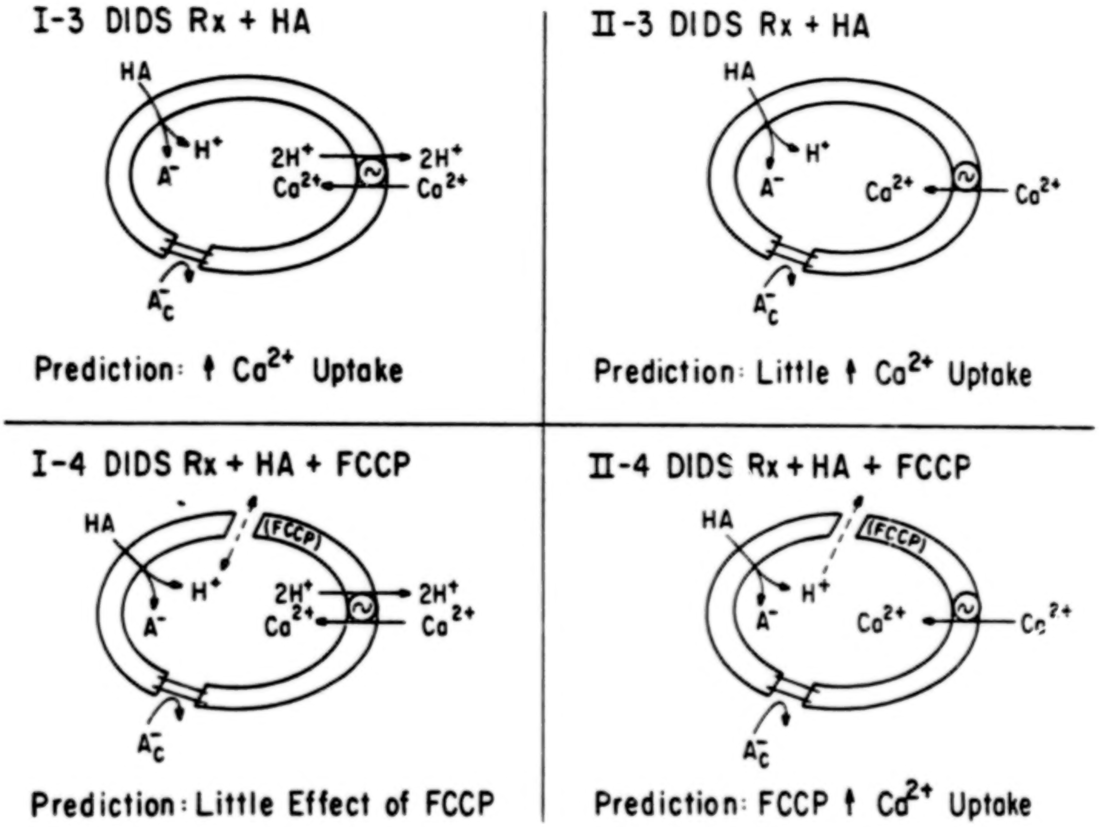
I-3 DIDS Rx + HA
HA
H+
A−
2H+
Ca2+
2H+
Ca2+
A−c
Prediction: ↑ Ca2+ Uptake
II-3 DIDS Rx + HA
HA
H+
A−
Ca2+
Ca2+
A−c
Prediction: Little ↑ Ca2+ Uptake
I-4 DIDS Rx + HA + FCCP
HA
(FCCP)
H+
A−
2H+
Ca2+
2H+
Ca2+
A−c
Prediction: Little Effect of FCCP
II-4 DIDS Rx + HA + FCCP
HA
(FCCP)
H+
A−
Ca2+
Ca2+
A−c
Prediction: FCCP ↑ Ca2+ Uptake

Figure 4. The predicted results of addition of weak acid (HA) upon Ca^{2+} uptake into inside-out, DIDS-treated, red-blood cell vesicles, if the calcium pump operates as a Ca^{2+}-H^+ exchange (left), or as an electrogenic pump (right).

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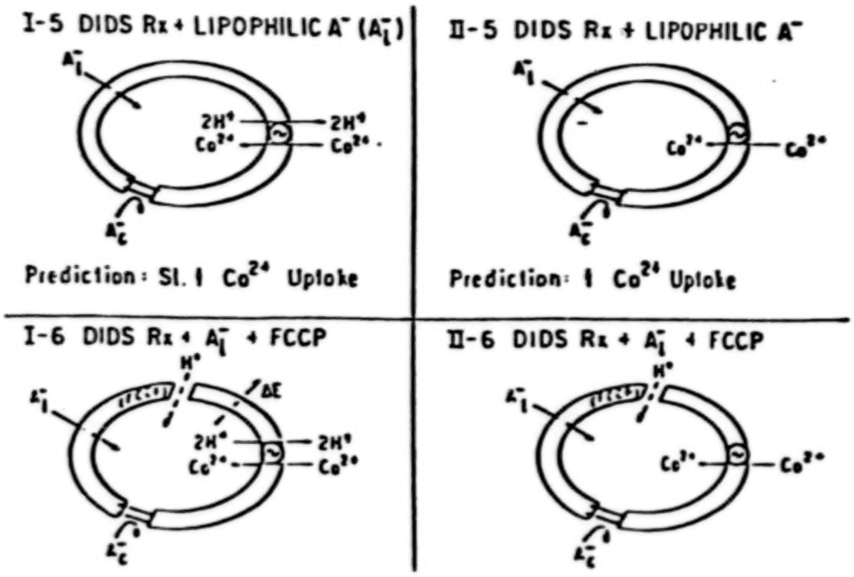
I-5 DIDS Rx + LIPOPHILIC A^- (A_l^-)
A_l^-
$2H^+$
Ca^{2+}
$2H^+$
Ca^{2+}
A_c^-
Prediction: Sl. ↑ Ca^{2+} Uptake
II-5 DIDS Rx + LIPOPHILIC A^-
A_l^-
Ca^{2+}
Ca^{2+}
A_c^-
Prediction: ↑ Ca^{2+} Uptake
I-6 DIDS Rx + A_l^- + FCCP
H^+
A_l^-
FCCP
ΔE
$2H^+$
Ca^{2+}
$2H^+$
Ca^{2+}
A_c^-
II-6 DIDS Rx + A_l^- + FCCP
H^+
A_l^-
FCCP
Ca^{2+}
Ca^{2+}
A_c^-

Figure 5. The predicted results of addition of lipophilic anion upon Ca^{2+} uptake in the presence or absence of FCCP.

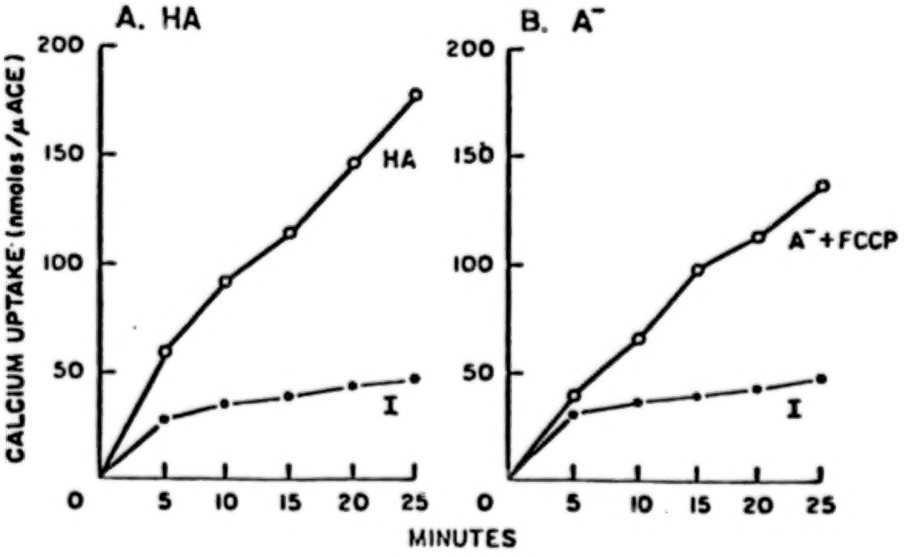

Figure 6. The effect of acetate (HA) and nitrate (A^-) plus FCCP upon calcium intake in DIDS inside-out vesicles from human red cells. The lower line (o-o) represents uptake in the presence of an inpermeant anion only. The upper lines (o-o) uptake in the presence of added HA or A^- FCCP.

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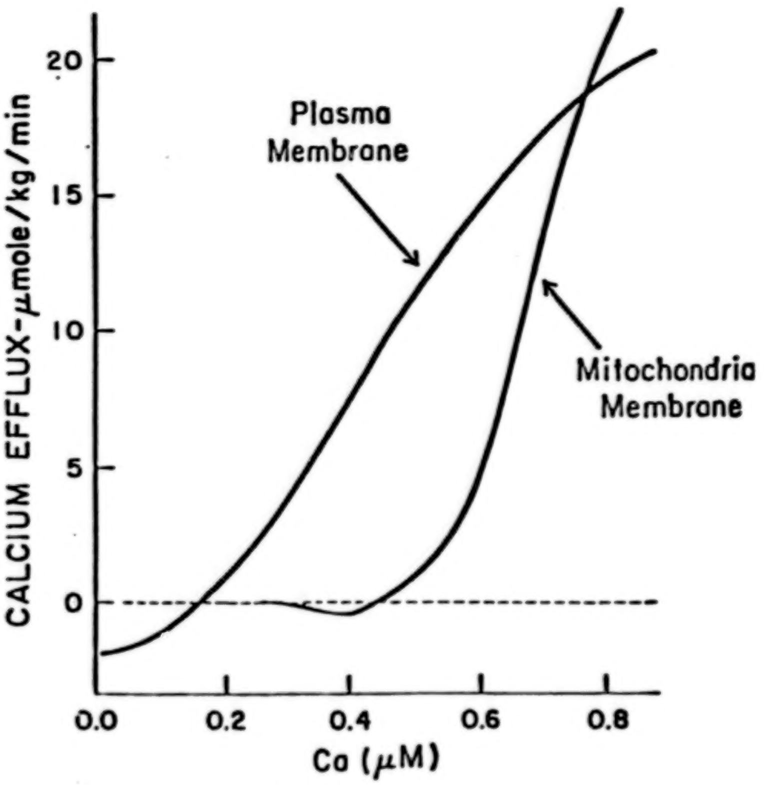

Figure 7. The rate of the calcium pump activity in plasma membrane or mitochondrial membrane as a function of the cytosolic calcium ion concentration.

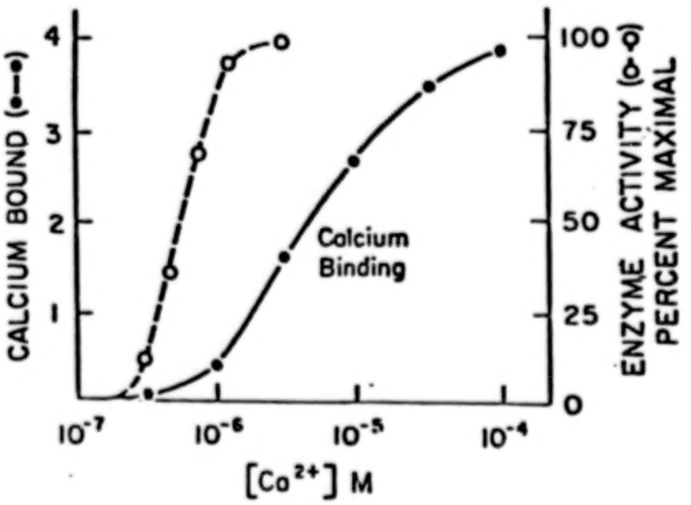

Figure 8. A comparison of the binding of calcium to calmodulin as a function of $[Ca^{2+}]$ (o-o) and of the activation of the calmodulin-activated enzyme phosphodiesterase.

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RASMUSSEN FIGURES

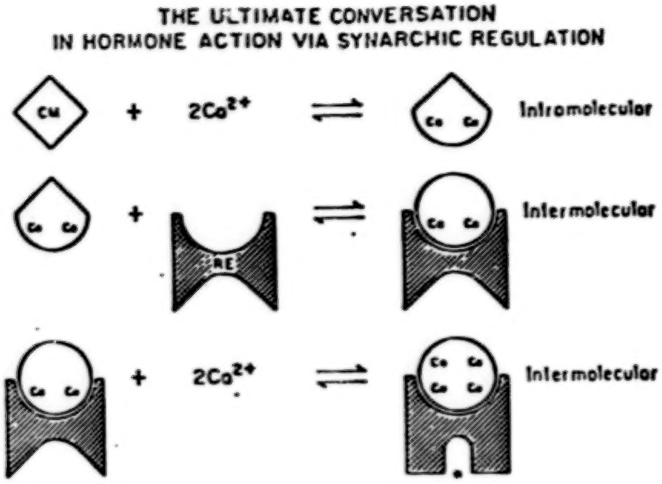
THE ULTIMATE CONVERSATION
IN HORMONE ACTION VIA SYNARCHIC REGULATION
CM
+
$2Ca^{2+}$
Ca Ca
Intramolecular
RE
Intermolecular
Intermolecular

Figure 9. A schematic representation of a three step, ordered, cooperative model if the interactions of Ca^{2+}, calmodulin and a response element.

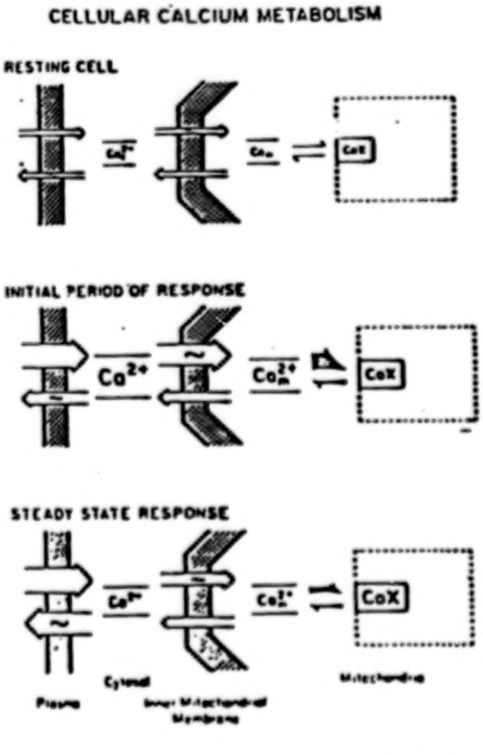
CELLULAR CALCIUM METABOLISM
RESTING CELL
Ca^{2+}
Ca_m
CaX
INITIAL PERIOD OF RESPONSE
Ca^{2+}
Ca_m^{2+}
CaX
STEADY STATE RESPONSE
Ca^{2+}
Ca_m^{2+}
CaX
Cytosol
Mitochondria
Plasma
Inner Mitochondrial Membrane

Figure 10. A schematic representation of a cellular calcium metabolism in a resting cell (top), when the cell is initially activated by a hormone, (middle) and when the cell displays a substantial response to the hormone (bottom).

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DR. ROUX: The first speaker in this session will be Dr. Peter Reed from Vanderbilt University, and the title of his talk is "Carboxylic Acid Ionophores as Probes of the Role of Calcium in Biological Systems."

DR. REED: When Stan called me up at the beginning of the summer, I was sitting around in my office wondering why I was in the department of pharmacology at a medical school, because the only drug I have ever used in my life, besides aspirin, is an antibiotic which I am going to talk to you a little bit about today called A-23187, produced by a Streptomyces strain.

Stan gave me something else to worry about, and that was what was I going to say that would be of interest to space biology people. I thought what might be useful -- and it may have been a little naive on my part -- is to talk a little bit about calcium ionophores, small molecules which can form lipophilic complexes with calcium, and transport that calcium across various membranes. I also wish to show you some of the pitfalls you can fall into using these agents and to describe sort of a survey of the effects of calcium ionophores. I am going to concentrate on one in particular, which I have had some fair investment in, A-23187. I will suggest one or two biochemical mechanisms which might underlie the very broad, general, diverse family of biological effects of this ionophore, focusing on arachidonic acid oxygenation, and the formation of a number of oxygenated metabolites of arachidonic acid, which has been studied in great intensity over the last five or ten years. They are involved in a whole number of bodily functions, perhaps, and I think their production may be regulated by calcium.

The message really is, although I think this message is known well by many of you, that a small molecule that can form complexes with calcium and that can disturb the calcium gradients that are very tightly maintained across the plasma membrane, as Dr. Rasmussen pointed out, a 10^4 concentration gradient, can be very useful to bring calcium into the cell, to turn on, or to inhibit various systems. When an ionophore such as this, one of these antibiotics, has an effect like this, it sort of implies that there is a role of calcium for regulating the system -- maybe not necessarily, but it has suggested in some cases a role for calcium where one hasn't been seen before.

I would like to end up by suggesting that the production of oxygenated metabolites of arachidonic acid may in fact be responsible for many of the effects that we see when we get calcium into a cell.

This is the structure of A-23187 (Figure 1). I came across this antibiotic ten years ago, studying its effects on mitochondrial function (1). At that time, there were other ionophorous antibiotics known, that is, other species of ion-transporting compounds that were produced by microorganisms, but none for divalent cations. This turned out to be the first which was able to form complexes with divalent cations, and they are lipophilic complexes, on the outside at least, so they can dissolve through membranes and carry the calcium through.

The molecule essentially could be considered to have three parts. One region is a substituted benzoxazole moiety. One of the oxygens on the carboxyl group and the nitrogen in this ring system are involved with liganding to a calcium atom which will sit in here. So these are two of the liganding species. There is a rigid spiroketo ring system, and then there is an alpha-ketopyrrole system. It is the oxygen on the keto group of the alpha-ketopyrrole system that forms a third liganding species.

Any of you who know anything about the chemistry of calcium know that it likes to ligand with between five to seven or more different oxygens or liganding atoms. So this ionophore forms a two-to-one complex with calcium. It is easier to see in this simplified structure (Figure 1). It is this keto oxygen, an oxygen in the carboxyl group, and this nitrogen which ligand to calcium. This is the first example of one of these agents which would ligand to a cation in a central cage by a nitrogen.

This is one-half of a stereo model, so it is perhaps a little difficult to see. But it shows you the two-to-one complex of A-23187. We can start with one of the molecules. There is the pyrrole ring and the alphaketo group. You can see it liganded. There is the central calcium in the center of this thing. There is the oxygen of the alpha-ketopyrrole group and the spiroketo ring system. There is the benzoxazole moiety of one of the antibiotic molecules which you can see liganded by the nitrogen, a member of that benzoxazole moiety, and then by one of the oxygens on the carboxyl group. So it is a threefold coordination from this molecule. The other molecule is liganded in the same fashion. There are hydrogen bonds between the two antibiotic complexes to form this stable complex.

It may not be immediately apparent from this picture, but the outside of this two-to-one complex is relatively hydrophobic or lipophilic. It is mostly carbon and hydrogen atoms. The inside is where the charged groups are, the negatively charged groups, the oxygens and the nitrogens. These replace the waters of solvation around the calcium molecule in solution, forming this two-to-one complex, at the surface of the membrane, probably. This then dissolves in the membrane to liberate calcium on the other side.

SPEAKER: What is the diameter of that complex?

DR. REED: Of the total complex, I don't know. I can't tell you. I can tell you the angstroms between the nitrogens and oxygens and the calciums, as well as something about the central cavity size in here and what this is optimally sized to fit in terms of cations.

SPEAKER: How big is it?

DR. REED: I have some data which suggests that the two-to-one complex that is formed by a whole host of different divalent cations and two A-23187s is optimally sized for zinc. Now, without going into a great amount of detail, unless you want me to, this is the work of Doug Pfeiffer with whom I collaborated when I was at the University of Wisconsin studying this antibiotic. He simply measured the ability of the ionophore to form the two-to-one complex and move into an organic phase. You put the ionophore in the toluene-butanol phase, calcium or whatever ion you want to consider in the aqueous phase -- you do this with the aqueous phase buffered at different pH's -- and you can generate an overall extraction constant, if you will, the overall formation constant of two antibiotic molecules with one divalent cation (2). That is plotted along the ordinate (Figure 2). The negative log of this overall extraction or formation constant, as it gets to smaller numbers, indicates that the complex is more favorably formed. Pfeiffer plotted this overall binding or extraction constant as a function of the ionic radius of the divalent cations we are talking about (Figure 2).

Look at only the solid symbols for the moment. This is A-23187. The data confirm rather well the transport properties of this ionophore when this has been looked at. That is, one would expect that barium would bind weakly -- barium is too big -- with an ionic radius of 1.35. It is too big for the two molecules to come together to hydrogen bond and to optimally fit around that cation. The ionophore is not good at transporting barium across lipid bilaters or across red cell membranes. It is better at transporting strontium. It is quite good at transporting calcium, about as good at transporting calcium as it is at transporting magnesium. This is the first warning I will make about using this calcium ionophore. The selectivity, at least for A-23187, is not very good between calcium and magnesium for complex formation, as shown here. In fact, I will show you some data in a minute that will show that this ionophore can distribute magnesium across membranes quite effectively.

As we get down to smaller cations of manganese and zinc, you find that the binding is even tighter. In some early studies we did with red cells, we were not able to get the ionophore to transport manganese appreciably (1, 3). It is probable that this fits too nicely into this cavity. The ionophore moves it into the membrane as a manganese complex, and it stays there. It doesn't really want to give it up, back to the aqueous solution. But transport studies with these other cations have not really been looked at. It would be an interesting possibility to study a little more thoroughly whether A-23187 can transport manganese or zinc.

The open circles are another ionophore, another carboxylic acid ionophore, as is A-23187. This one is called X-537A. They get these numbers from the drug companies, who number them according to batch, et cetera. X-537A has quite a different spectrum for binding cations. It is quite effective to transport barium. It is best for barium and least effective for calcium and magnesium (1).

I don't want to let you think that the only calcium ionophores are A-23187 and X-537A. There is a newer one that has come out over the last several years, ionomycin (1). It has an entirely different structure, although it is still a carboxylic acid, and forms one-to-one complexes with calcium, because it has two anionic groups on the molecule. It seems to move faster and be a little more effective concentration-wise. It perhaps has a little better selectivity for calcium, for magnesium, but all of these divalent cation ionophores will interact with magnesium in biological systems, or at least have the potential to, as well as calcium.

Now, we have been interested for some years in the acute inflammatory cell, the polymorphonuclear leukocyte, and we study guinea pig and human neutrophils, or polymorphonuclear leukocytes, and have studied the effect of the ionophore A-23187 on them. I am showing you this as a model system, just to indicate the kind of ion fluxes which can occur when A-23187 is added to a cell with extracellular calcium in the medium. The cells in this case are the neutrophil leukocytes. They are incubated in a sucrose medium, which is buffered, containing two millimolar calcium.

We have plotted here the nanomoles of calcium taken up upon addition of the ionophore. That is shown in Figure 3. Here are the nanomoles of cations, hydrogen, magnesium, and potassium, released, expressed as per milligram protein, shown here as a function of time.

The initial exchange which this ionophore produces across, I think, most biological membranes is a calcium-hydrogen exchange. This is certainly true in the white cell. It is true in the red cell (3). It hasn't been looked at in a lot of systems. But initially here, in the first half-minute or so, it is a one-calcium-in/two-hydrogen-out exchange.

I should make the point that the ionophore produces only electroneutral exchanges. Since it is a carboxylic acid, and is bringing calcium into the cell, it will become, after it releases the calcium inside, an anion, and it needs to bring something out or it won't get through the membrane. What it likes to do quite well, even though the hydrogen concentration is only 10^{-7}M or so in a cell, is bring hydrogens out. Many of the carboxylic ionophores do this.

So this calcium-specific agent is very capable of causing a pH change in your cells. If you add it to your cells and you stimulate a process -- almost all the literature employs this type of approach -- you say, "Aha, this process is calcium-dependent." Probably that is the case. But it certainly could be due to the internal alkalinization of the cell caused by hydrogen ion loss.

DR. RASMUSSEN: Have you actually measured the intracellular pH?

DR. REED: No. I should tell you how I measured these species. These three ions (calcium, magnesium, potassium) were measured with atomic absorption, simply, the content in the cell before or after treatment. This is simply the hydrogen ion concentration, in a weakly buffered medium. So I don't know, in fact, that this actually produces internal alkalinization. I do know there is a fairly large amount of hydrogen ion added to the extracellular medium, measurable with a glass electrode. I think other people have, using pH-sensitive dyes, indicated that this ionophore can cause internal alkalinization.

After this has taken place, we begin to see a magnesium loss (Figure 3). This represents a fairly substantial loss of the intracellular magnesium. This might not occur if the extracellular medium had two or three millimolar magnesium, as many buffers do. We see, very interestingly, potassium loss from these cells, which is really quite marked in terms of their total potassium content. This can be seen in a sucrose-containing medium, as here, as well as in a high sodium-containing medium. We saw this type of a phenomenon initially in studies with an erythrocyte (3), where it turned out that after the ionophore brought calcium into the cell -- the calcium inside stimulated a massive potassium loss. This is the so-called Gardos phenomenon that was described in 1958 (4), without any ionophores at all. Simply when red cells accumulate calcium, they lose potassium. We are seeing it here, with the ionophore bringing calcium in, causing secondary changes in the potassium content of the cells.

It is possible that the ionophore is forming potassium complexes, but when one tries to look for complex formation of A-23187 in vitro with monovalent cations, it is quite weak in complex formation (1, 2). It will transport sodium into various cells, if you have no calcium and no magnesium around (1). It will next go to sodium. But you can't show direct potassium transport under most circumstances.

Well, the message is that what I am calling a calcium-selective ionophore really perturbs a number of other ion gradients within cells. Almost universally, people who use A-23187 pay no attention whatsoever to what other ions might have been disturbed within the cells. It probably does turn out that calcium is the major ion that is producing the effect that they look at.

Table 1 represents some eight years of work, since the ionophore first became available from Eli Lilly. It summarizes in a very general way, some biological effects of the ionophore A-23187 on a host of different cell systems or tissues (5). I can be much more specific, if any of you want me to, about this after the talk.

Most of these systems for the effects of the ionophore require calcium in the extracellular medium. Many of these changes occur within one cell type. So they probably are secondary to the ionophore bringing calcium into the cell. Some of these are not surprising at all. We have known that muscle contraction is dependent on calcium for a period of time, and it really was no surprise that smooth muscle, striated muscle, cardiac muscle, all can be shown to be stimulated to contract with the ionophore or to increase contractility.

Exocytosis of hormones, enzymes, and neurotransmitters bound in intracelluar granules triggered by the ionophore is also no real surprise, because a role for calcium in this system has been known for a long time.

Stimulation of secretion of ions and water, for example, by the lachrymal gland or the ileum or the colon, may be a little bit more of a surprise. A-23187, in a calcium-dependent manner, causes marked stimulation of chloride or sodium fluxes in these tissues.

With transformation and division of cells, there has been a suggested role for calcium in these systems for a long time. A-23187 can cause transformation, stimulation of division of lymphocytes, for example.

An example of a system in which there really still is, to my knowledge, no well-defined role for calcium is in the transport of certain substrates. The ionophore will increase the rate of transport of glucose or certain amino acids across certain cells, such as the thymocyte. In certain cultured cells it will generally decrease the rate of uptake of neurotransmitters. This may indicate a possible area that ought to be examined in the future: the transport of substrates by carrier-mediated mechanisms is a calcium-dependent or perhaps a calcium- and calmodulin-dependent system.

Well, the ionophore will cause, usually, depolarization, which often is dependent on sodium, as well as calcium in the extracellular medium, will stimulate the synthesis of DNA and RNA in cells where it causes division and transformation, quite often will inhibit the synthesis of proteins in various cultured cell lines. Whether this indicates a true role for calcium getting into a cell and inhibiting protein synthesis is a question.

Then there is a huge area of effects of the ionophore on metabolism, turnover of phospholipids within membranes, arachidonic acid metabolism, which I am going to focus on shortly, alterations in intracellular messengers, such as cyclic AMP and cyclic GMP, enhanced protein phosphorylation, which I will also address a little more thoroughly in a minute, changes in ATP levels (usually a drop), reports of activation in certain systems of glycogenolysis, glycolysis, enhanced hexose monophosphate activity. And there are some 30 or 40 enzymes which have been reported to be activated based on the production of what one would assume to be their products in different tissues by the ionophore A-23187 (5).

What might be some common possible biochemical mechanisms underlying all of these diverse effects of bringing calcium into a cell? Figure 4 illustrates this point. Don't pay any attention to how many calciums I have binding to the calmodulin, because I don't know this either. I want to remake the point that calcium going in is obligatorily exchanged for other cations coming out with the ionophore A-23187. The M is monesin, another carboxylic ionophore which causes sodium-hydrogen exchanges. But A-23187 has also been shown to release calcium from intracellular sites. Specifically in the sperm cell, it has been shown to be capable of moving calcium out of, probably, mitochondria into the cytoplasm. I would hypothesize that it might also, added to certain cells, release calcium from sarcoplasm reticulum or other storage vesicles, perhaps even release calcium from membrane-bound sites. For example, in the neutrophil leukocyte there is a membrane-bound, chlorotetracycline-sensitive pool of calcium which is discharged when you add the ionophore in the absence of any extracellular calcium. So some studies can be done by adding the ionophore to a tissue or a cell, with no calcium outside, and one can still see an effect. It may be that that effect is because the ionophore is releasing calcium from some sorts of intracellular pools and raising cytoplasmic calcium, even though you have no calcium in the medium (5).

Well, we have heard a lot of calmodulin dogma. It is probable that most of the calcium effects, or many of them, within cells are due to formation of calcium-calmodulin complexes, with subsequent activation of many different enzymes, such as the calcium ATPase, which pumps the calcium back out of the cell, which Dr. Rasmussen talked about so nicely a few minutes ago, protein kinase -- calcium-dependent protein kinases may be plasma membrane or soluble enzymes -- adenyl cyclase, phospholipase, phosphodiesterase, phosphorylase kinase, myosin light-chain kinase (Figure 4). These are just some of the enzymes that were listed in Milt Cormier's talk.

I would like to suggest that many of calcium's effects, and many of A-23187's effects therefore, on biological systems may be due to protein phosphorylation and phospholipase A-2 activation. I am going to spend most of the rest of the talk on this particular part.

This is all data from other people's work, where A-23187 has been added to these cells or tissues and the phosphorylation of very specific polypeptides has been measured (Table 2). It is worth making the point that in these tissues the protein which gets phosphorylated by stimulating the tissue with A-23187 is very often a protein that is phosphorylated when one stimulates the tissue with a physiological agent, instead of an ionophore which breaks down the plasma membrane barrier to calcium. For example, in platelets these same two polypeptides which are increasingly phosphorylated by A-23187 -- if one adds collagen to platelets, you see the same two polypeptides become phosphorylated. In the mast cell these same three peptides are phosphorylated when one treats the mast cell with compound 48/80, which is a histamine releasing agent. In the hepatocyte both glucagon and norepinephrine, through an alpha effect, cause phosphorylation of these four polypeptides, as well as quite a few others (5).

So protein phosphorylation which we haven't studied this yet, but we intend to, is probably a major way that calcium enters the cell and regulates cell function. Perhaps through protein phosphorylation, A-23187 produces activation of phospholipase A and formation of all the products that result from oxygenation of arachidonic acid.

It was known for some years, or suggested for some years, which phospholipases, particularly phospholipase A-2, which cleaves a fatty acid from the number two position of various phospholipids, is calcium-dependent and, it was thought by many people, calcium-regulated. So Howard Kuapp, a graduate student in our department at Vanderbilt, came to me in 1976 or so and said, "You work with this marvelous compound which brings calcium into cells. Why don't we see if it stimulates arachidonic acid metabolism?" We and many others began similar studies and published them at the same time (6).

Now, the arachidonic acid cascade at that time was best worked out from studies with platelets (Figure 5). It simply is that arachidonic acid is mainly bound in the form of phospholipids, either phosphatidyl inositol or phosphatidyl choline, in plasma membranes; that is, cells have no appreciable levels of free arachidonic acid within them. Upon activation of a phospholipase A-2, arachidonate is released into the cytoplasm or near other membranes and is metabolized, depending on the tissue, to a whole host of different compounds. Initially, an enzyme called cyclooxygenase metabolizes arachidonate to prostaglandins called G-2 and H-2, which are cyclic endoperoxides. These are active intermediates with a fairly short half-life, something on the order of 20 seconds in an aqueous medium. They serve as the starting compounds for the highly active compounds, thromboxane A-2. It is a very powerful vasoconstricting agent produced by platelets and is involved with platelet aggregation. Prostacyclin produced by vascular endothelium is a very potent inhibitor of platelet aggregation and it inhibits the production of thromboxane A-2 -- and what you perhaps are more familiar with, the standard, earlier described arachidonate products, prostaglandins E-2, F-2 alpha, and D-2. Again, depending on the tissue, the profile of the oxygenated metabolites will be different. For example, in the renal medulla it releases primarily E-2, a little F-2 alpha, and a little D-2. The mast cell makes primaily D-2.

The differences in these compounds are whether there is a keto up here or a hydroxyl here, two hydroxyls, or whether the keto and hydroxyls are reversed. They are not very marked differences, and yet the enzymatic profile of different cells is different because they tend to produce one or another primarily. Platelets produce very large amounts of thromboxane A-2, which has a half-life of the order of 10 seconds or less in aqueous medium. It very rapidly changes nonezymatically to thromboxane B-2. But while thromboxone A-2 is around, released from the platelet or within the platelet, it is potentially a very powerful agent. Released from the platelet, it is a very potent vasoconstrictor.

So this is the cyclooxygenase pathway. It incorporates molecular oxygen in a cyclic endoperoxide, further oxygenates at other positions. Cyclooxygenase, as many of you may know, is inhibited by aspirin and indomethacin, two nonsteroidal antiinflammatory agents. It is probable that many of the effects of aspirin and indomethacin in human beings, as pharmacological agents, are due to inhibition of the release of many of these products in response to various stimuli.

Now, at the time we began this study, there was another pathway known from platelets to metabolize arachidonic acid, but not very much was known about it, a lipoxygenase pathway, the pathway on the left (Figure 5). Aspirin and indomethacin do not inhibit this pathway. In the platelets what happens is this lipoxygenase -- an enzyme which has been studied quite a lot in plants, but not too much in mammalian systems -- inserts a hydroperoxy-group at carbon 12 of the arachidonic acid to form 12-hydroperoxy eicosatetranoic acid. So it is called HPETE, hydroperoxy eicosatetranoic acid. This, rather rapidly, is metabolized by peroxidases to the monohydroxy fatty acid, HETE. So this is 12-HETE, which is the major lipoxygenase product of platelets. Other cell types produce different HETES, for example 5-HETE. The function of these products was not known for a long while. It turns out, upon testing, that some of these agents are potent chemoattractants. That is, 12-HETE released from the platelet, will cause white blood cells to migrate to that site.

So these compounds have a wide variety of different types of functions. The kidney produces primarily prostglandins E, D and F2 alpha, and they may be involved in the release of things such as renin or in renal transport. I think nobody really knows. There is a vasoconstrictor (thromboxane A-2), inhibitor of vasoconstriction, in fact vasodilator (prostacyclin), a chemoattractant (HETE). We will see this gets worse instead of better.

This enzyme, lipoxygenase, as I said, is not inhibited by aspirin or indomethacin. It is inhibited by a compound called ETYA, which is the acetylenic acid analog of arachidonate.

We found in some of our early studies that activation of the acute inflammatory cell, the neutrophil, by different stimuli, such as A-23187 or a chemotactic peptide, N-formyl-methionyl-phenylalanine, was inhibited by ETYA (7). That is, you could turn the cell on by these compounds to show chemotaxis, to release certain enzymes, to generate superoxide anion. All of these things they do to try to kill bacteria. You could inhibit these functions if you put in ETYA, which would really block both of the pathways for arachidonic acid metabolism, and perhaps have other effects, too. Aspirin and indomethacin had no effect on these cells, so that it looked to us like there might be some lipoxygenase pathway for the metabolism of arachidonate in these cells that was the key to activation of a neutrophil leukocyte.

So we labeled that arachidonate in these cells with tritiated arachidonic acid, washed the cells, incubated them in a bicarbonate buffered medium with calcium, without and with ionophore A-23187 (7). At the end of the incubation, we spun the cells out of the medium, in this case, added deuterated internal standards of all of these different compounds -- we didn't do all of these in one experiment -- subsequently extracted from the medium with ethylacetate the different products, cleaned them up, put them over reverse phase liquid chromatography, and subsequently analyzed them by gas chromatography mass spectrometry to get the actual amount of these products produced by the cells.

Table 3 shows the products formed from endogenous arachidonate that we prelabeled. The ionophore causes rather gross stimulation of the production of most of the cyclooxygenase metabolites. The major one that was released that was a cyclooxygenase product was thromboxane B-2, and this did not come from contaminating platelets. We were able to eliminate that. But there was a marked stimulation in thromboxane B-2.

All of the HETEs are lipoxygenase products. That is, the peroxy group was inserted in different parts of the molecule. You can see that the leukocyte we worked with mainly produces 5-HETE, not 12 like the platelets do. Again, this is tissue specificity. The ionophore causes marked stimulation of this. So it looked to us as if -- and this doesn't occur in the absence of calcium -- the ionophore is stimulating the release of arachidonic acid in these cells, and this arachidonate is being metabolized both by cyclooxygenase and by lipoxygenase. Since our data with inhibitors had suggested that some one of these lipoxygenase products were involved in regulating the cell, the only one that was really a candidate was 5-HETE, the only one that was really produced very much. When we looked at whether or not the drug which inhibited the cell's function, ETYA, had an effect on the production of this compound, it made it go up instead of down. So this was not the key regulatory molecule involved in these cells' function.

At about this time, Bengt Samuelsson and his colleagues at the Karlinsky Institute really had been doing a major amount of work with the arachidonic acid pathway, studying leukocytes, and they described an entirely new group of compounds, now called the leukotrienes (8). Now, Figure 6 is just that side of the previous pathway that we saw, with arachidonic acid being metabolized in the leukocyte by a 5-lipoxygenase to 5-hydroperoxy-ETE, this being metabolized by peroxidase to the 5-HETE. This compound is chemotactic. It does certain other things. But it was not the compound which was regulating our cells.

Samuelsson described the leukotrienes which are doubly oxygenated metabolites of arachidonic acid. They derive from a 5,6-epoxide intermediate called leukotriene A. The only ones which are probably enzymatically derived from this are leukotriene B-4, which is 5S-12R-dihydroxy-eicosatetranoic acid -- it is stereochemically pure; it is certainly coming from enzymatic hydrolysis -- and what are called the SRSs, slow reacting substances of anaphylaxis. For years, people had known that during asthma attacks, and during other anaphylactic reactions, substances which were lipid-like and which contained sulfur were released from sites in the lung causing the bronchial constriction, et cetera. With the description of this pathway of metabolism for arachidonic acid by Samuelsson, it became clear that the SRSs, the slow reacting substances of anaphylaxis, the bronchoconstrictors, were in fact leukotrienes. What they have is a peptide attached to the number six position of the leukotriene. Leukotriene C-4 has glutathione, which is shown here as SR. If you clip off the terminal glutamate, it is leukotriene D-4, and if you clip off the next amino acid it is E-4. C-4 and D-4 are powerful bronchoconstrictor agents. Furthermore, in certain species, these agents stimulate the production of thromboxane, which is another powerful vasoconstrictor.

Well, in our cells, looking about for one of these which might be the active metabolite, we focused on leukotriene B-4, since it was produced enzymatically. We have been able to show, as others have, that when this is added to the neutrophil leukocyte, one can mimic the effect of turning on the cells with a chemotactic peptide or an ionophore or other stimuli, partially, not completely. We are currently looking at leukotriene A-4 and its effects on the cells.

This is a little hard to do. First of all, you are adding these things to the outside of the cell, and that is not the way the cell is going to generate them. It is going to be generating it inside. Secondly, leukotriene A-4 has a half-life of six seconds or less at pH 7. So it is a difficult compound to work with -- for me to work with at any rate.

We asked the question, did the ionophore stimulate production of these compounds, and the answer was yes. The leukotrienes have a conjugated triene system. They have a characteristic absorbance at 270 to 280 nanometers because of this conjugated triene. The HETEs, because of a conjugated diene, have a characteristic UV absorbance at 235. So one can use UV absorbance as a simple way, without going all through the business of GCMS and deuterated standards, which don't exist yet for these compounds, to measure their production.

Simply, we incubated the neutrophil leukocytes with or without the ionophore, extracted the cells, added a standard prostaglandin B-2, which I haven't shown you the structure of. It migrates similar to these dihydroxy acids, and it has a UV absorption spectrum because of a conjugated triene system.

What you see is, a reverse phase HPLC system, with the most polar compounds coming off the front. Without the ionophore, there was no release of products by the cells (7). When they were stimulated with A-23187, we see several peaks, and we are able to point to the fact that two of these peaks are nonenzymatically derived from leukotriene A. The ionophore also stimulated leukotriene B, the enzymatically derived one, two more nonenzymatically derived compounds, the delta lactone of leukotriene B-4 and 5-HETE (7).

So A-23187, in a calcium-dependent manner, stimulates the production of the leukotrienes, as well as all of these other prostaglandins (7). Furthermore, if one adds exogenous arachidonic acid to these cells, and adds no ionophore, you don't get much metabolism of this exogenous arachidonate. But when you add the ionophore, now you see production of these compounds, particularly leukotriene B-4, the biologically active one, and 5-HETE in much larger amounts. So the ionophore is doing more than one thing to the arachidonate cascade. It almost certainly is releasing endogenous arachidonate and allowing it to flow through the different enzymatic pathways that exist in the different cells. Furthermore, it is stimulating, in some fashion, the production of, specifically, the leukotriene pathways and stimulating the production of these metabolites from added arachidonic acid. The mechanism of the latter is not known at all.

Well, lest you think that this is just something which occurs in the white blood cell, I would just like to finish by showing the same phenomenon in a variety of different cell lines. A lot of them are blood cells, platelets, neutrophil, the mast cell, and macrophages. The ionophores used are almost always A-23187 or lasalocid, which is another name for X-537A. In one case, two other ionophores that are available, which carry only monovalent cations, were able to work as well as those which carried the divalent cations. Largely, when it has been examined, it is only those that carry divalent cations that will work.

The products are very different depending on the tissue. Platelets release mostly thromboxane A-2, a very powerful aggregatory substance. The rat renal medulla releases prostaglandins E-2, F-2 alpha and D-2. It doesn't release thromboxane and it doesn't release, so far as has been reported, leukotrienes. Rat stomach and trachea release prostaglandin E-2. We have gone through the different compounds that the neutrophil releases. The eosinophil has a different profile as does the mouse macrophage. The epithelial cells release primarily prostacyclin (prostaglandin I-2). The thyroid gland releases prostaglandins E-2 and F-2 alpha. A whole host of different types of cells that have been incubated with A+23187 have been shown to release the slow reacting substances of anaphylaxis, measured simply as effects on biological tissues (slow contraction of bronchial muscle, for example) or were measured as the purified leukotriene C or D in different conditions.

To summarize the message I am trying to get across, A-23187 and other calcium ionophores can be useful agents to manipulate the calcium within cells, either bringing it from outside in or perhaps releasing it from within. But you have to be cautious because there is a whole host of other changes, such as potassium loss, magnesium loss, hydrogen loss, with potential changes in intracellular pH which can contribute very definitely to the final effect you measure. You may not simply be getting calcium in the cell and calmodulin mediated effects.

Many of the effects are, no doubt, through this pathway, by activating different enzymes or by activating the production of messengers within the cell, the standard messengers, cyclic AMP and cyclic GMP. I would suggest that it is possible, at least, that the arachidonate metabolites in many cell systems may be intracellular messengers. Many of the effects of these compounds that I have talked about today are effects on other tissues. For example, the release of prostacyclin from endothelial cells to produce vasodilation or the release of thromboxane A-2 from a platelet to cause powerful vasoconstriction. But these compounds are believed to have effects within the cells that produce them as well. This is quite clear from the neutrophil leukocyte.

So many of the effects of getting calcium into a cell, I feel, may be mediated by changes in its messengers. In many tissues we may be seeing in the future that the arachidonic acid metabolites may, in fact, be the business end of what is going on, just before we measure our effect, at least in many disease states.

Thank you.

DR. ROUX: Any questions?

DR. BIKLE: It is reasonable to think that hormones which modulate calcium across membranes might do that by somehow activating endogenous ionophores. You have done some work in this area. I am wondering if you could give us some information on what your concepts are on that.

DR. REED: Well, I got very excited when we started this prostaglandin business way back, because I thought, here are some compounds which at least have more than one oxygen atom in them, that potentially could ligand to calcium ions. I thought that maybe these oxygenated metabolites of arachidonate might be endogenous ionophores. That is, they weren't sitting around; they were produced from phospholipids near the membranes when they were needed. They could do the ion fluxes. Then they would be metabolized to inactive products.

With the different prostaglandins, a lot of people have shown that they can behave as ionophores and I have, too. I have never had the courage to publish it, because I don't believe they behave as ionophores in physiological situations. You can make phosphatidic acid behave as an ionophore. I think that is baloney.

DR. BIKLE: Why do you reject these?

DR. REED: Well, it is just intuitively unpleasing to me, I think. If you look at Gerald Weismann's work (9), the concentrations of phosphatidic acid you need are 10^{-5}-10^{-4} molar. He uses phospholipid liposomes with a dye inside that changes color when you bring calcium in, and he adds the different putative ionophores outside. They are pretty high concentrations. Well, there are a lot of phospholipids in the membrane, so maybe. But would you need this phospholipid to flip through the membrane? It has been shown in most systems that that doesn't occur, in fact.

I don't particularly care for the idea of phospholipids as ionophores.

The latest story, which I perhaps should have pointed out, is that the compound, leukotriene B-4 -- three hydroxys and a carboxy -- people have started saying that it is an ionophore (9). I think the data for this is a little more believable. I am still not sure I like it all that well. That is, arachidonic acid would be released, metabolized to this compound, which would then be near membranes, and could alter calcium gradients to produce all the things which actually this does.

The argument is that leukotriene B-4 is stereospecific. It is 5S-12R. This is the wrong configuration of the double bond. This is an early slide. It needs to be cis-trans-trans. If you take all trans, instead of cis-trans-trans, no ionophore activity. If you take 5S-12S all trans, no ionophore activity. So the ability to carry calcium into these liposomes is highly stereospecific. The concentrations required are a couple orders of magnitude greater than A-23187. You are up in the micromolar range. That is pretty high.

I guess that is all I want to say about endogenous ionophores, at least lipid-like compounds.

DR. CLELAND: Do any of the effects of A-23187 that you described occur in the presence of chlorpromazine or other calmodulin inhibitors?

DR. REED: Well, let's ask it the other way around: Are any of A-23187's effects inhibited by chlorpromazine? Yes, they are. But we and other people have shown that if you add, in some systems, only as much as 10 to 25 micromolar of these phenothiazines, you can inhibit the ionophore's ability to transport calcium pretty well. So you may see an inhibition of effect, but it could be, I think, due to nonspecific effects or effects on the ionophore's ability to transport calcium. We have done this several times, and I just don't like the data with the phenothiazines.

DR. WATTERSON: Two points. One, you dodged the point about calmodulin and phenothiazines.

But I want to ask you about phospholipase A-2. As you well know, there is a phospholipase A-2 in which the crystal structure is determined, and it is well known what the molecular mechanism of action is, not by a calcium-modulated mechanism, but by an active site involved in catalysis. Now, there is controversy over the phospholipase A-2 that is supposed to be intracellular in its calcium-binding properties, as opposed to models put forth by Dr. Rasmussen. Could you make some comments on the data with phospholipase A-2 that would be involved in this type of regulation?

DR. REED: The kind of regulation I envision here is not that the calcium is required at the catalytic site. I won't quarrel with that. But maybe the story that Hirata (10) has developed recently is the case. He has developed this in the neutrophil leukocyte. It is simply that the phospholipid-bound arachidonic acid is clipped off by phospholipase A-2, but that this in the cell is kept in an inactive form -- it can't do its thing — by a protein he calls lipomodulin, not to be confused with calmodulin. So that inhibits that. It can't work.

Now, he has been able to present some data that phosphorylation of lipomodulin -- and he thinks it is done by a calcium-dependent protein kinase -- deinhibits its effect on phospholipase. So if you could phosphorylate this inhibitory protein, you would free up the phospholipase, and it could start chewing on arachidonate, and you would get all this metabolism.

The kinase that does this, he has shown in vitro, in test-tubes, can be a cyclic A-dependent protein kinase. But he shows that certain stimuli cause fluxes of calcium into the cell, which he thinks activate the calcium-dependent protein kinase.

That is the way I envision what may be going on, but I don't know. I realize there is controversy over the way this enzyme might be regulated by calcium. I think it is pretty clear that you can turn on phospholipase activity -- Howard Rasmussen will back me up -- in a whole host of tissues with a calcium ionophore, not with a monovalent cation ionophore. So it is not just a detergent effect.

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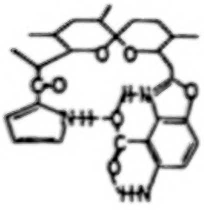
Figure 1



A23187

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Figure 2

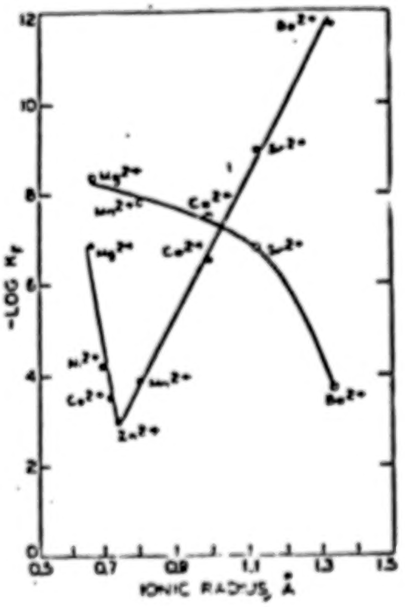

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Figure 3

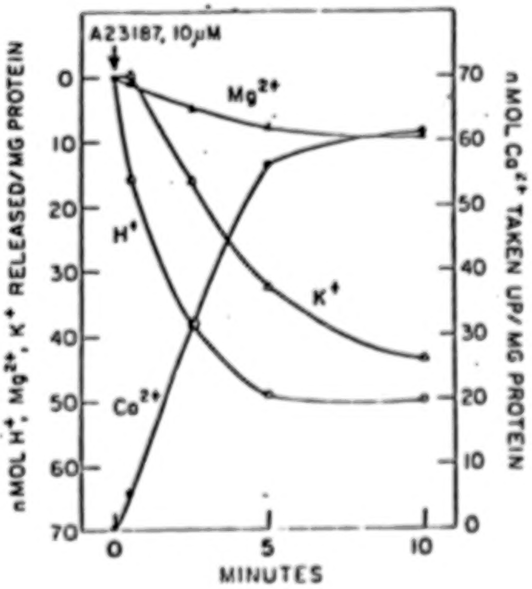

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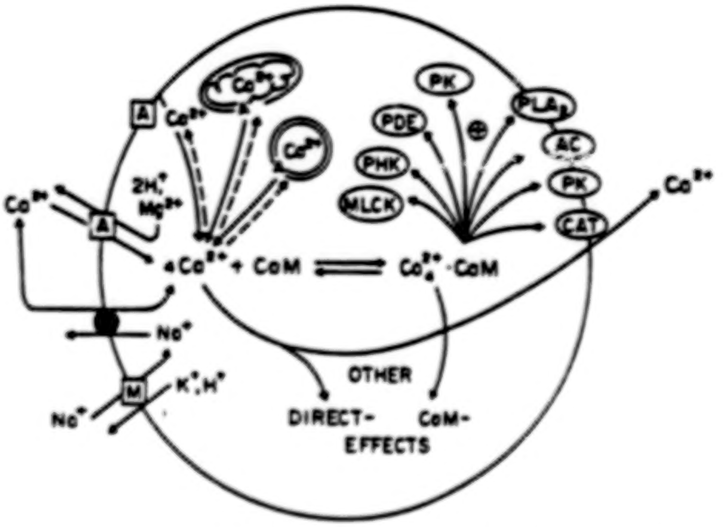
Figure 4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Ca^{2+}
Ca^{2+}
Ca^{2+}
PK
PLA_2
PDE
⊕
AC
PHK
PK
MLCK
CAT
Ca^{2+}
Ca^{2+}
$2H^+$, Mg^{2+}
A
$4Ca^{2+} + CaM \rightleftharpoons Ca_4^{2+}\cdot CaM$
Na^+
OTHER
M
K^+, H^+
Na^+
DIRECT- CaM-
EFFECTS

The possible relationship of some calcium-dependent ionophore effects to calmodulin-activated enzymes. Symbols are: A, A23187; M, monesin or other monovalent polyether antibiotics. Abbreviations are: CaM, calmodulin; CAT, Ca^{2+}-stimulated ATPase; PK, protein kinase; AC, adenylate cyclase; PLA_2, phospholipase A2; PDE, phosphodiesterase; PHK, phosphorylase kinase and MLCK, myosin light-chain kinase. Intracellular calcium storage sites are depicted in the upper left quadrant of the cell as calcium bound to the cytoplasmic surface of the plasma membrane or sequestered in mitochondria, reticulum, or possibly granules. Dashed lines are meant to indicate mechanisms for lowering cytoplasmic free Ca^{2+}, in addition to extrusion from the cell by the ATPase.

Chapter 5 in Polyether Antibiotics Volume 1: Biology Ed.
J. W. Westley Marcel Dekker, Inc. N. Y. and Basel, p. 195.

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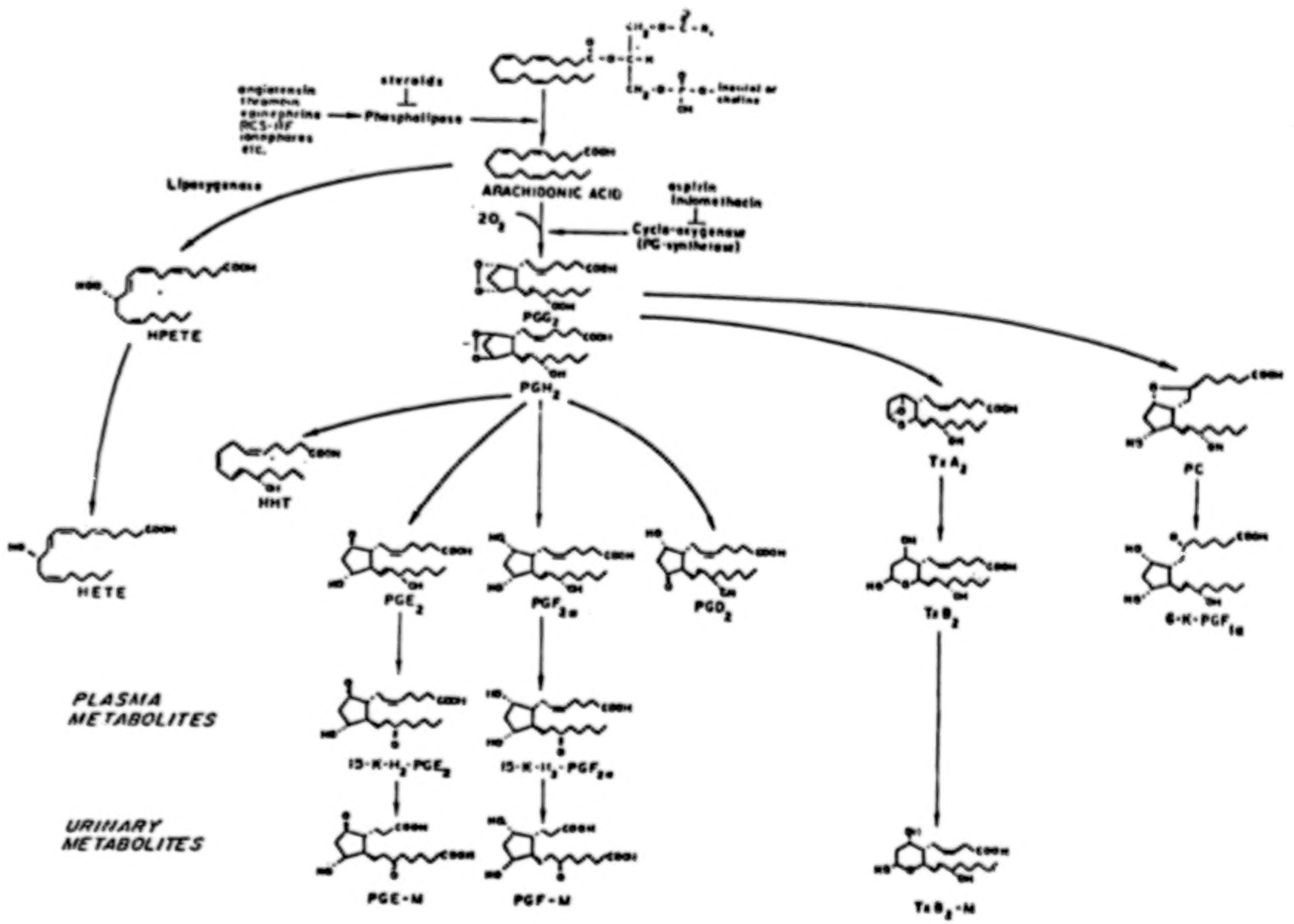
angiotensin
thrombin
epinephrine
RCS-RF
ionophores
etc.
steroids
Phospholipase
Lipoxygenase
ARACHIDONIC ACID
aspirin
indomethacin
Cyclo-oxygenase
(PG-synthetase)
2O₂
PGG₂
PGH₂
HPETE
HETE
HHT
PGE₂
PGF₂α
PGD₂
TxA₂
TxB₂
TxB₂-M
PC
6-K-PGF₁α
15-K-H₂-PGE₂
15-K-H₂-PGF₂α
PGE-M
PGF-M
PLASMA METABOLITES
URINARY METABOLITES

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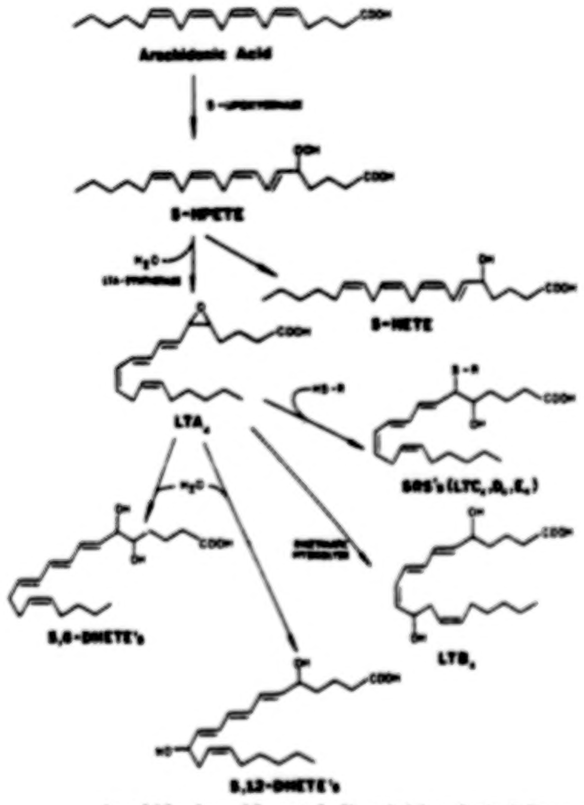
Figure 6


COOH
Arachidonic Acid
5-lipoxygenase
OOH
COOH
5-HPETE
H_2O
OH
COOH
5-HETE
O
COOH
LTA$_4$
HS-R
S-R
COOH
OH
SRS's (LTC$_4$, D$_4$, E$_4$)
H_2O
OH
COOH
OH
5,6-DHETE's
OH
COOH
OH
LTB$_4$
OH
COOH
HO
5,12-DHETE's

Arachidonic acid metabolism initiated via 5-lipoxygenase. *DHETE's*, dihydroxyeicosatetraenoic acids; *SRS's*, slow reacting substances; *LTA$_4$*, leukotriene A$_4$.

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Reed Tables

Table 1

SOME EFFECTS OF IONOPHORE A23187 ON CELLS OR TISSUES

CONTRACTION of muscle and non-muscle cells; changes in cell shape: ↑
EXOCYTOSIS of hormones, enzymes and neurotransmitters: ↑
SECRETION OF IONS AND WATER: usually ↑
TRANSFORMATION AND DIVISION OF CELLS: usually ↑
TRANSPORT OF SUBSTRATES: glucose, amino acids ↑; uridine, neurotransmitters ↓
MEMBRANE POTENTIAL CHANGES: usually depolarization, sometimes hyperpolarization
SYNTHESIS OF MACROMOLECULES: DNA, RNA usually ↑; proteins, glycosaminoglycans ↓
METABOLISM: phospholipid turnover, arachidonic acid metabolism ↑ ; cyclic AMP, cyclic GMP, protein phosphorylation ↑; ATP levels ↓; glycogenolysis, glycolysis, hexosemonophosphate shunt ↑ ; gluconeogenesis ↑ or ↓
ENZYME ACTIVITY: usually ↑

↑ indicates stimulation, ↓ indicates inhibition

Table 2

STIMULATION OF PROTEIN PHOSPHORYLATION BY A23187

Cell or Tissue	Phosphorylated Protein (M_rX10-3)
Parotid gland fragments	total protein
Human platelets	20, 47
Mast cell, serosal	47, 59, 68, (↓78); 45-53 (IgE receptor)
Hepatocyte	49, 52, 61, 93
Cerebral cortex synaptosomes	41-43
Torpedo, cholinergic synaptosomes	100

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Table 3

OXYGENATION PRODUCTS OF ENDOGENOUS ARACHIDONIC ACID RELEASED TO THE EXTRACELLULAR MEDIUM BY NEUTROPHIL LEUKOCYTES AT REST OR STIMULATED BY A23187

Product	Control	+ 10 µ M A23187
	ng/mg protein	
PGE_2	0.06 ± 0.03	0.51 ± 0.08
$PGF_{2\alpha}$	0.13 ± 0.01	0.40 ± 0.07
TXB_2	0.17 ± 0.05	3.83 ± 0.33
5-HETE	0.83 ± 0.17	13.58 ± 1.92
8-HETE	0.03	0.07
9-HETE	0.04	0.04
11-HETE	0.04	0.10
12-HETE	0.03	0.05
15-HETE	0.22	0.30

Neutrophils were incubated with 1-2 µM [^{3}H]-arachidonic acid to label endogenous phospholipids and, after washing, incubated for 15 min with 10 µ M A23187 or the appropriate amount of solvent as control. Deuterated internal standards were added, the cells were sedimented, and the incubation medium was extracted twice with ethyl acetate. Products were separated by reverse phase high performance liquid chromatography and quantitated by gas chromatography-mass spectrometry. References to further description of methods are found in reference 11. Values given are the mean ± standard error of the mean of 3-8 experiments, or the average of two experiments where no standard error is given. PG, prostaglandins; TX, thromboxane.

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DR. ROUX: The last speaker on this morning's program is Dr. Roger Tsien from the University of California, Berkeley. The title of his talk will be "Measurement and Control of Free Calcium Inside Small Intact Cells."

DR. TSIEN: I guess I am here to put in a partisan advertisement for the importance of actually measuring the intracellular free calcium concentration. Lots of people in this room have done fine work in analyzing effects on cell function of, say, varying extracellular calcium, measuring all sorts of calcium 45 fluxes, measuring total cell calcium, adding calcium ionophores or adding putative blockers of calcium entry.

Other people have also -- also many of them in this room -- done an equally fine job of finding calcium-binding proteins in tissues, looking at effects of putative calmodulin blockers on cell function, or even developing in vitro models where the cell membrane is permeabilized or removed and then looked at how the proteins extracted from cells, say, are affected by buffer changes in calcium.

Yet both sorts of data, I feel, have a serious incompleteness until one actually measures the intracellular free calcium in the cell. That is the central parmmeter which links the first type of experiment to the second. I would like to remind you, of course, that calmodulin, if you think about it, doesn't know anything about nanomoles of calcium per milligram of total cell protein. What it can sense on the molecular scale is its local free calcium.

Just very briefly, why has free calcium been so difficult to measure until now? The answer is there are two problems. First, there is so little of it. We have heard many times dogma says it is 100 nanomolar or 10^{-7} molar. That is very little compared to the free concentrations of the other cations, such as potassium, sodium, magnesium. Magnesium is the worst offender because it is a millimolar, approximately, free, and that is 10,000 times more. You need pretty good selectivity -- better than A-23187 has, anyway -- to tell the difference between those two. The second problem, which I will get to, is that anything which did have the selectivity to bind calcium at a 100 nanomolar then couldn't get into the cell. Well, fortunately, there are some chemical solutions to these problems. I will start first with the problem of calcium-to-magnesium selectivity. How do we get it chemically?

Back when I started thinking about this in '75, there was only one really good substance known to bind calcium specifically in the presence of magnesium, potassium, sodium at intracellular levels, and that was the chemical everyone used then, and still does now, for buffering calcium, EGTA. That is its structure (Figure 1). But, of course, EGTA doesn't indicate calcium. It has no chromophore of any sort. It occurred to me by various bizarre turns of mind that the way to turn this stuff into an indicator was to attach benzene rings at these positions, to make something like this, and initially the simplest possible, with just hydrogen atoms, just two simple benzene rings. What I would do by this means is preserve the geometry of the binding site, two nitrogens, two oxygens, four carboxylates, pretty well preserved. But then there would be a chromophore, and not only would the chromophore be somewhere on the molecule, it would be very close to the binding site, or intimately involved -- as intimately as you could have. When you build molecular models, you can see that the conjugation of the nitrogen lone pairs will be removed when calcium binds.

As a side benefit, EGTA is well known to be very pH-sensitive, because in normal pH 7 medium it has protons bound on the nitrogens. That is,

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it has two pKa's up at 8 or 9. So they are protonated at pH 7. In order for the calcium to come on, the protons have to be kicked off. That is an equilibrium process, so the pH is very strongly affecting the calcium binding. It also slows the thing down very markedly. So EGTA binds calcium very much more slowly than the diffusion control rate. When you put benzene rings on, the nitrogens becomes aromatic amines instead of aliphatic. It could be predicted that the pKa's would drop. So this stuff was going to be nonprotonated at pH 7, which would mean that it wouldn't be pH-sensitive anymore and it would be a lot faster.

So I thought this was worth a try, and secretly, against the wishes of my adviser -- well, my adviser didn't know; otherwise, he would have forbidden it -- I made the stuff, and it fortunately had the properties as expected. The calcium affinity of this stuff is almost exactly 100 nanomole. It has a very weak magnesium affinity, better than five orders of magnitude discrimination, simply because we have maintained the steric geometry of this binding site. Why mess up a good thing? The pKa's are below 7, so it isn't protonated, it is faster at binding and unbinding, and it has an optical signal. The spectrum of the free form has peaks in the near-UV. When calcium binds, it shifts all these peaks that way, or decreases the long-wavelength absorbance, just as predicted if you removed the lone pairs.

Well, this is all well and good, but, of course, this substance, called BAPTA -- an acronym for a long chemical name -- is useless as an intracellular indicator, because all these absorbances are just where aromatic amino acids absorb, and you would never see anything in the cell. It could be used as a buffer, a pH-independent fast buffer, but that wasn't really all that exciting. Obviously, to get this thing out to longer wavelengths, one wants to increase the length of the conjugated chain, which is how you make dyes go to longer wavelengths. I will show you a couple examples of how this can be done.

On one of the earlier ones I did, I just put an azo group on, a very common way of making dyes out of anilines. This is orange dye to which calcium binds and depresses the long wavelength or shifts it to shorter wavelength, depending on how you want to think about it, and turns into a very pale yellow stuff when the calcium is bound. There is a lower affinity for calcium because of the electron withdrawing power of that group. On another one we put an even bigger chromophore on. Now you have a blue dye absorbing at 600nM. When you add calcium, it shifts into a sort of orange-pink color.

One reason I show you these is that people have tended to think of the dye that actually we have used most as an entity by itself, as if it came out of isolation, the "quin" dyes. Basically, the common feature of these dyes is the tetracarboxylate structure derived from EGTA (1). I would prefer to call them tetracarboxylate dyes. I will get to the quin dye. It happens, for historical reasons, to be the one we most use. But I consider it a chemical deadend. While these have their imperfections, the ones based on the original structure are where future progress is probably going to be made. So I don't want people getting fixated on thinking there is just one dye called quin 2, which you will hear about next, just because it does happen, for various reasons, to be the one we have used most so far in these early studies.

Figure 2 shows you quin 2 itself. The reason it is called quin is because of quinoline. The 2 becomes the second one I made. The first one didn't have the methoxy group. In this case, the conjugation is extended a little bit, and mostly sideways. There has been one change. Instead of an oxygen here bridging the two aromatics, there is a nitrogen there, which causes certain problems, but it was necessary for this particular molecule. would like to now go into more detail on the properties of this stuff (2). One thing good about it is that it is strongly fluorescent, and that fluorescence is highly sensitive to free calcium. Now, this is just a simple dye in vitro in a calcium buffer at 37 degrees in a simulated ionic intracellular environment. It has the right high potassium, low sodium, and one millimolar free magnesium. What we have done is titrate the dye out by adding calcium to the buffer to change the free calciums, and these are the free calcium levels, zero, 10, 20, and so on. It is very sensitive in the region around where we expect resting level to be, 100 nanomolar or so. This is the emission spectrum. This is the excitation of the fluorescence (Figure 3).

We now take these amplitudes at the peak and plot them against free calcium to get this plot, which is a log scale of calcium and a simple one-to-one binding curve (Figure 4). It is simple one-to-one because I gave it eight sites, which is enough to keep anything happy, eight chelating sites. EGTA is a one-to-one chelator, so it is not surprising that this thing also is. It makes life much easier when calibrating to have it with this simple chemistry. I mention this because many of the other calcium indicators are rather more complicated. The open circles are the data you saw from the previous slide with one millimolar magnesium. When you analyze this, you find that there is an effective Kd of 115 nanomolar, right about exactly where the resting level is going to be, so we have maximum sensitivity at the resting level. But if the calcium goes too high, like above a micromolar, we must admit, this dye is not going to see very much, because it is practically saturated. So that is one limitation. Its sensitive region is right around resting. Beyond a certain point, it will get saturated.

Incidentally, Figure 4 shows the effect of the magnesium. If we do the whole series again without the magnesium, you find a somewhat higher dissociation constant. That is zero magnesium giving you 60 nanomolar. Curiously, it doesn't affect the maximal fluorescence, with maximal calcium, nor the minimum. That is useful, that magnesium doesn't itself change the fluorescence property of any individual molecule; it just competes slightly at the binding isite. If we know magnesium, to a moderate degree, we can get the calcium fairly accurate. We don't have to know magnesium too well, but it is better to know it if you want to be quantitative, the more quantitative you want to be about calcium. Fortunately, we were able to measure the free magnesium in several of the cells that we are actually interested in, and so we know it directly instead of just guessing.

I would like just very briefly to emphasize the difference from chlortetracycline, which Peter Hepler told us about. If you have an intracellular organelle, it will pick up typically a high amount of calcium from the cytoplasm, and the place that chlortetracycline is generating a lot of fluorescence is probably on the inside face of this membrane, because it has to sit on a membrane, and it needs a lot of calcium. The quin 2 sits in the cytoplasm. It is not hydrophobic. It is practically insoluble in organic solvents. In various tests we find it doesn't bind to membranes or proteins to a significant degree. So CTC is measuring some sort of membrane-enclosed calcium, whereas quin 2 is a hydrophilic solution dye out in the cytoplasm.

Well, I just mentioned that there is going to be a problem getting this stuff into cells without microinjection, because it is so hydrophilic and non-hydrophobic that it is not going to just permeate membranes easily. This held us back for quite a while. What we really wanted was some way of making it membrane-permeable. That would mean masking the negative charges. Unfortunately, those negative charges are what permit it to bind calcium. So this seemed somewhat incompatible, until you realize that what you really want is a temporarily membrane-permeable derivative that can cross the membrane and then turn itself back into the calcium-binding form, with four negative charges, which will trap it there, just where you want it.

When you think of uncharged, nonpolar derivatives of carboxylic acids, the obvious thing that comes to mind is esters. An unfortunate problem is that this is a rather unnatural acid that the cell has never seen before. Cells do have esterases, but they like to chop esters that they have seen before. This one it hasn't, and so, if you just make a simple methyl ethyl ester, it doesn't hydrolyze. It can get into things, but then it doesn't bind calcium usefully.

So what to do? Well, fortunately, there is a trick to be found, which is to make a mixed ester. Here is one of the carboxylates coming from the quin 2:$RCOO^-$. Remember, there are four of them. But just for the moment, here is one. You make this sort of mixed ester $RCOOCH_2OCOCH_3$, which has one methylene group sandwiched between two. This one is just an ordinary acetate. The whole unit $-CH_2OCOCH_3$ is an acetoxy methyl group. It so happens that the cell, for reasons best known to itself, knows how to chop acetate esters. What it does is it hydrolyzes this to $RCOOCH_2OH$ and CH_3COOH. Then there is the fortunate organic chemical property of any sort of ester in which there is one hydroxy directly on the methylene that it falls apart spontaneously, no enzymes required. It releases that and methylene glycol. Both of these are low molecular weight substances that can diffuse out of the cell. That leaves the one we want, $RCOO^-$. Of course, we have to do this four times for the four ester groups, and we have to wait long enough for them all to fall off to get the full calcium binding back. But this, in principle, is the way to do it, and, fortunately, it works (3). It is quite general, as far as we can tell, for getting otherwise impermeant carboxylic acids into cells, not just the quin 2 ones. This may be of some long-term benefit to other people. So the procedure, really, now couldn't be much simpler. The chemistry may seem complicated, but you don't have to understand it to use it. What you do is you take the cells and expose them to the ester and wait. The stuff diffuses in and gets hydrolyzed. Depending on the conditions and cell type, we usually use 15 minutes to two hours operationally.

Where does the dye go? It goes where the esterase activity is. It diffuses, apparently, everywhere, as far as we know. But where it finds the esterase, there it begins to be chewed up and then it is trapped. Once the carboxylates start being uncovered, it can't move across membranes anymore.

The final intracellular concentration you can control just by controlling the ester concentration, the number of cells, and so on. Typically, you wind up with from 30 to 60 or so percent of all the ester that you put in suspension inside the cells, because it is continually being trapped there. Some stay outside, perhaps because there are some dead cells that release esterase, and there is a small amount of nonenzymatic hydrolysis and so on. That doesn't really matter. In the end, you wash the cells to get rid of the hydrolyzed stuff outside that doesn't bind to the cells, so you can wash it out. You are left with a population of cells with the dye trapped inside.

Incidentally, the esterase seems to be in the cytoplasm, and that is just good luck to us. You can find that out in two ways. First, you can fractionate cells and look: Do mitochondria have esterase? Does ER have it, and so on? And the answer is very little. The esterase activity seems to be in the supernatant of a cell prep. Or you can actually load in intact cells, then fractionate and see where it is. There are various ways of doing that. Again, the answer seems to be that it is in the cytoplasm. It also does get into the nucleus, apparently, because there are big nuclear pores. But it seems to permeate freely without either concentrating or being excluded from the nucleus. So that is okay to us, because we don't see that there will be a calcium gradient across there. For us, the nucleus is just an extension of the cytoplasmic space.

Chelator loading via ester, so far, we have tried on the following cells, and there is at least some loading into all of them: red cells, mast cells, lymphocytes, platelets, adrenal medulla, Erlich ascites, Yashita ascites, thymoma cells -- the last three were cancer types -- macrophages, neutrophils, pancreatic beta and insulinoma cells, gastric mucosal glands, basal cells from mouse skin, toad bladder, frog retinal rods, sea urchin eggs, frog skeletal muscle. With the last two of those, the sea urchin and the frog muscle, it is very sluggish. Generally, we find that the lower the temperature one is forced to work at, the slower, and sea water doesn't help, because the ester is by then so hydrophobic it can hardly dissolve in sea water. There is some uptake. You can just detect it. But it is not very comfortable at the moment. I think changing the ester group, changing acetate or so on to other things, may eventually help that. So I don't think it is a permanent problem, but at the moment it is a slight obstacle.

How do we actually measure the free intracellular calcium? We have the dye and we have the method for getting it in.

Here is the loading procedure (Figure 5). The ester has a different fluorescence spectrum than the final stuff. So if you want to see on a new prep whether it hydrolyzes, you can watch it in the fluorimeter as it changes. This is how you know how long it takes. It is the easiest way of following how long it takes to get loading. Once you have worked it out on a given cell type, it is not worth bothering to record it routinely.

This is the sort of skeleton experiment. We have a population of loaded cells, drop the dye inside them. They are sitting in normal medium, one millimolar external calcium. So what is the free calcium? Well, in principle, if we had complete knowledge of how many cells we had and the sensitivity of the fluorimeter, all we would have to do is look at that and say, yes, we know how much. But we don't even know how much dye is in for sure a priori. So the easiest way to find out what calcium this is signalling is to take that amount of dye and force it to known calcium levels. That establishes the calibration curve. The easiest way to do that is to lyse the cells. We lyse them in this case with digitonin. Triton also works. Anything will do. You get a much higher fluorescence. This is because now the dye is exposed to one millimolar calcium.

In this particular case (Figure 6), we wanted to be fanatical, and we actually titrated the calcium to two intermediate values in this lysate, 200 and 100 nanomolar, or maintaining the magnesium in pH under intracellular light conditions. Finally, we went to zero calcium by putting in a lot of EGTA and raising the pH. If you look, this fluorescence corresponds to something between 100 and 200 nanomolar. Therefore, you get an estimate of

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what the free calcium is. It turns out that quantitatively the easiest thing to do is to measure the difference between here and here, and here and here, plug it into a very simple formula. This interval divided by this interval times 115 nanomolars gives you the free calcium of any fluorescence that you have. In subsequent slides, that is how we derive the calibration marks you will see from the fluorescences, just by that formula.

Incidentally, this shows the fluorescence of cells that have no dye in them at all. It changes practically negligibly with calcium, with lysis or any of these procedures. We go through a dummy run with no dye in the cells. It just about sits here, though we make a small correction for it.

DR. JAFFE: What is the fluorescence due to in the zero calcium in there?

DR. TSIEN: Because when you strip the calcium off the molecule, it still has a little fluorescence. It has one-sixth of its maximal fluorescence, about. The difference between here and here, which is due to calcium-free dye, should be one-fifth or one-sixth different from here all the way up to there. It does usually work out pretty well. This shows that the dye has the right affinity. It has come back to about 115 nanomolar. This is some confirmation and relief to us that the ester process really did regenerate good dye, not crippled dye, that it did come back to its in vitro properties.

I already touched on the point of localization, where is the signal coming from. I don't really have time to go into it, though. If anyone wants, I will try. Basically, we believe it comes from dye in cytoplasm. So it is measuring cytoplasmic free calcium.

I also don't really have time to discuss toxicity in detail. Obviously, one wants to see, has this treatment injured the cells? Our results, in general, find that it does not, at least when you put one, two, or three millimolar on. Higher than that you do begin to see some toxic effects, the exact cause of which we are not sure. But there is a nice range near a millimolar which in our hands doesn't give toxic effects, at least on short term. I wouldn't swear, if you kept one millimolar in them for a year, that they wouldn't show something. We haven't done that sort of testing. We are interested in short-term physiological experiments.

I do want to comment a little bit more, however, on buffering. After all, we have put in, in this case, something like 0.6 millimolar of a high-affinity EGTA-like buffer into the cytoplasm. You might wonder, is this itself affecting the calcium that we are going to want to measure? Well, if so, you would predict that the more you put in, the lower the calcium reading, apparent calcium, would be. You don't find any such dependence. When you actually plot apparent calcium reading versus the quin 2 content, it is not a slope like that, which would be consistent with the buffer lowering the calcium. It is not significantly different from zero. The nominal linear regression is actually slightly positive.

Well, how can this be? The answer seems to be, when you work it out, that as the quin 2 goes in, that plasma membrane, which Dr. Rasmussen talks about, of course is a clever beast; it wants to maintain the calcium at its normal resting, and now that the buffer is inside picking up calcium, it just lets in a little more to compensate. We can verify that, for example, by showing that if you have three millimolar quin 2 inside the cells, the cells now have about one-and-a-half millimolar total calcium more than they are used to having. Presumably, that is the one-and-a-half that is needed to saturate the quin 2, while yet keeping the cell's own buffers as happy as they were before.

A little bit more confirmation comes if we deliberately frustrate the cell's homeostasis. After all, they are taking on board calcium. Well, what if we give them no calcium to take, by putting them in a calcium-free medium? Well, in that case you do get very low calcium levels. In this case, for example, we put in two millimolar, a little more dye, and we start it at 16 nanomolar. Very low calcium, well below normal. Here we gave it back the normal calcium it wanted, one millimolar, and up went the fluorescence to a level, if anything, slightly supernormal, probably because the cells are a little leaky after an hour-and-a-half in low-calcium medium. I am not surprised it is a little bit high (Figure 7).

Notice how fast it is, two minutes or so. Normally, the loading takes an hour. So if you give it calcium throughout, you can imagine that it would easily maintain its calcium with practically no deficit, just the faintest possible one. It would have its feedback good enough to maintain calcium. Incidentally, this high calcium permeability -- we have better experiments on red cells -- argues that at these very low calcium levels, at least in red cells -- these are lymphocytes, but if we go to red cells, where you can do it better; I don't have the slide -- you actually open a calcium permeability that wasn't there in the normal cell. That calcium permeability shuts off as the calcium reaches a normal level, which is a bit curious. You figure if it already has a 10,000-fold gradient, why should a 30,000-fold gradient make it any different? Well, something in there actually senses it and seems to open a permeability, just another line of defense for the plasma membrane to hold the calcium steady. That just shows that if you add the calcium back in little dribs and drabs, it eventually gets to the same level.

Now I would like to give you some examples of what you can actually do with it when analyzing stimulated cells. You have a stimulus put on a cell. You think the calcium goes up inside, and then there is a response which some of you already know the phenomenology about. Can we decide whether it is calcium-stimulated? Instead of boring those knowing this and myself with yet another story of a T lymphocyte, which I have done too many times and has been published already (2, 4), I have chosen to take my examples for this presentation from B lymphocytes and platelets. This is somewhat more recent work. The B lymphocytes, I should mention, were primarily done by Tullio Pozzan in Italy, partly in Cambridge and then in Italy (5).

I first have to tell you a bit about what a B lymphocyte does. How do you stimulate it, and what does it respond? Well, it does many things. Of course, its main job is to make antibody. But we are focusing on one little aspect of that, which is that when you bind an agonist to the surface receptors of a B lymphocyte, and it has surface immunoglobulin on it as its receptor, when you bind antiimmunoglobulin onto it, somehow the cell knows to pull all these receptors into what is called a cap. They were randomly distributed on the cell surface before. You put the ligand on, and they cross-link somehow, and they pull them all into one area. This is a sort of elementary form of cell motility. It happens to be shown on a membrane, but presumably there is some sort of actin or whatnot -- and there is EM evidence for it -- pulling these things together.

Now, people already knew from the sort of standard type of calcium physiology methods that, if you put tracer calcium in cells while you do this, you find that there is a measurable calcium efflux. However, their first sampling point was a minute, and by then it was fully developed. They also knew that chlorpromazine would block the capping. Back in those days, they thought chlorpromazine was a simple local anesthetic, and just called it local anesthetic. In small print, you found it as chlorpromazine. That blocked it, and therefore they said that is what it is doing, it is blocking calcium movements across membranes, because of the cationic amphiphilic amine. Nowadays, we might think of other possibilities for chlorpromazine.

Finally, they put A-23187 on in high doses, above a micromolar. What it did is it caused sort of a general disorganization of everything. These things sort of all went writhing around, and even formed caps, after which what had already been made by cross-linking would then be dispersed.

I don't quite know how to interpret that, but they said that is excellent evidence for the involvement of calcium. So all together they said, what this does, somehow, is it really causes calcium to be released from internal stores -- that explains that -- there is some calcium entry, and all together that starts the cell motility machinery into action -- because everyone "knows" the cell motility has got to be calcium-dependent -- and that is the response.

That is a very reasonable idea, and it is sort of typical of the sort of inferences you can make when you don't measure the free calcium. You are going to get the impression already that we don't quite feel that this is the entire picture. I guess I am probably the only person in this symposium who is actually showing evidence that calcium doesn't do something. So I will give the answer away.

This is an experiment on mixed spleen lymphocytes. One reason I have chosen this problem is that it shows you the difficulties of quin 2. Of course, it goes into all sorts of cells. It turns out that mouse spleen lymphocytes are only half B cells. Half of them are T cells. The T cells don't even bind the ligand, and we have every reason to presume that they are not interested. They are just passive bystanders. Nevertheless, they are sitting there in the fluorescence beam, and so we measure some composite between it. Fortunately, we can measure in every experiment what is the proportion of the B cells, the ones that actually bind to ligand, because you can get the ligand fluoresced and labeled and so on, if we can make various assumptions like the T cells and the B cells start with the same resting level -- that is reasonable, I think -- and that the T cells just sit there, and it is just these two components. In that case you can correct for the fact that you have dilution. But it does warn you, if you have a heterogeneous cell population, at the moment we just look at the average.

Well, here (Figure 8) we put on rabbit anti-mouse immunoglobulin. That is what that acronym RaMIG stands for. That is the ligand, in this case. The fluorescence goes up right away. We noticed good time resolution. It is about as soon as you can get the lid of the fluorimeter back down, or within ten seconds maybe. So, yes, there is a rise in intracellular free calcium. Right away you can say that. It is sort of sustained, but eventually it does fall back down, after waiting long enough.

Incidentally, if you actually measure the progress of capping on a parallel sample -- if you take aliquots out - you find that it has just barely begun at one minute. So it is clear right away that, whatever it is, capping did not cause the rise in calcium, because the calcium came first in time. But this still quite allows the rise in calcium to have caused the capping.

That is all well and good. So the earlier workers were right. There is a rise in intracellular free calcium.

In this experiment (Figure 9) what we did is we put the EGTA into the external medium just before stimulating, and took the external free calcium down to something under 100 nanomolar, just before the beginning of each trace. There is a slow creep-down because it just leaked, or pumping out. But here is an interesting point. Here we put on the ligand, and again the calcium goes up. Now, in this case there is practically no calcium outside to come in. There is less than 100 nanomolar outside. So this has to be coming from some sort of internal binding site. Yes, the workers were correct; calcium is released from an internal store as well. It turns out, when you analyze things, that this rise is somewhat smaller than the earlier one. The earlier one was done in normal calcium, and that shows the plasma membrane does contribute as well. So, yes, just as Dr. Rasmussen said, we have a combination of internal release and entry from the external environment.

Now, what these show is what happens when you put more chelator in. I said previously that the chelator didn't change the steady-state calcium, but that is after the cells had many minutes, or hours even, to adjust things back to steady state. But what you do expect, if you put a lot of buffer in, is that you will temporarily damp transients, fast transients. I think this one was something like one millimolar dye (Figure 9, Panel A) and three millimolar dye (Figure 9, Panel B) and you can sort of guess that the signal might be about three times smaller. You do a couple of these and you can more or less convince yourself that at this region there is an approximate inverse relation between the amount of dye you have in and the apparent rise in free calcium.

But that means there is always a fixed amount of calcium being released. Using that proportionality ratio, it turns out to look like about one-quarter millimole per liter of cells being released inside the B cells. It happens to match very nicely what the tracer people said. Yes, there is about a quarter millimole liter released. I mention this because it shows that you can use this dye, which you might have thought just measured free calcium, since it is a buffer and since you can control how much it is, and you know the stoichimetry, to measure movements of total calcium, without having to use tracers, spin down the cells, worry about extracellular carryover, all sorts of things. You can occasionally use it to measure total fluxes.

Where is this calcium located inside the cell? What sort of internal store is it? Under certain occasional preparations -- we don't know why some do and some don't -- you can actually see those two phases, the internal release and the sustained entry. You can see them separate on the time scale, not just by changing external calcium. These were done in one normal calcium (Figure 10). This is a very low dye concentration. The first thing you get is this spike, which we attribute to be the internal release, then followed by a sustained plateau, which actually falls back down a bit. When you put a lot of dye in, what you do is decrease this first amplitude in the normal way, because the fixed amount of calcium just being dumped — there is more dye, and it doesn't raise the free calcium as much. Then you get this slower sustained level, which comes to the same free calcium in the end. This is the plasma membrane sort of slowly chugging along, letting in calcium bit by bit, titrating it. So this has all the characteristics of being the sustained release, and sometimes you can see them separated in time.

Going back to where were the internal stores, this is in calcium-free medium. When we want to look at internal stores, we tend to use calcium-free medium to avoid plasma membrane entry. We put A-23187 on in quite a low dose (Figure 11). This is just 40 nanomolar. People who had worked on this previously were using well over a micromolar, I believe, in the feeling that that is what was necessary to raise calcium. This is just 40 nanomolar, and you get a very nice rise. After the A-23187 has gone on, you put on the proper, or closer to physiological, ligand, and nothing happens. This is unable to release anything from internal stores. We would say that this is probably because they have all been depleted. A-23187 has already let out what is going to be let out.

If we do it the other way around, you can get some release from here. You put the A-23187 on, and you get a smaller further release, not as big as this one, but sort of the rest. We don't quite know where this is. It could be other compartments in the B cell or it could be the T cells, which before were just passively sitting around. Of course, A-23187 has no specificity in the way the immunoglobulin does.

Curiously, it shows that, for this coupling between the immunoglobulin and whateven mysterious internal organelle lets the calcium out, that coupling requires ATP. The immunoglobulin also doesn't work here, and the ATP is low. In some confirmation, we have put oligomycin -- that doesn't release calcium from mitochondria, but also poisons the ATPase -- this also lowers the ATP in the cells, and again the immunoglobulin has no effect. So somehow we think the coupling here needs ATP. It is an energy-dependent process. What it is we would really like to know, but we don't.

To finally get back to the main question, sort of, that started it all, what about the cell motility? Does that depend on calcium, the intracellular free calcium rising? Well, you get some hint of it from this experiment, if you actually look at the cells, take aliquots out. I am afraid I don't have photomicrographs. You will have to take my word that you can assess capping, or at least Tullio Pozzan could assess capping. With the addition of A-23187 we have a nice big calcium transient. You find there is no capping there. But when RaMIG comes on, it doesn't cause any further transient, and the capping occurs then.

Now, we wouldn't have done it on this experiment because there isn't enough time for separation. There is only two minutes, and capping itself takes a few minutes to develop. But if you space it out, you can find A-23187 at this low dose, which doesn't kill the ATP the way the high doses do, doesn't do anything to the capping or to motility. But RaMIG can, even when there is no rise in calcium detectable.

We can do this in another way. Here I am going to use that trick of loading the cell with chelator in calcium-free medium. That way you can achieve very low calciums. In this case it was something like 20 nanomolar, at this point. Probably the intracellular stores are a little bit depleted too by this long exposure to low calcium. We put on the agonist, practically no rise in calcium, and it certainly doesn't get back up to 50 percent saturation. So it stayed well below rest throughout here. If you look at cells which you give time to see whether they develop capping, yes, they do. They do cap even though their calcium has been kept down to 20 nanomolar, five times less than the resting level.

In this particular -- just a fluorescence record -- Tullio put back the calcium, and it goes back up as usual, as in other lymphocytes. It recovers very rapidly.

DR. BIKLE: Was the capping normal?

DR. TSIEN: As far as he could see, yes. Controls were 80 percent, and these went by about 72 or 75 percent, which is maybe a tiny bit down. But, of course, the cells had been treated a long time in nonphysiological medium. So we consider that to be as good as control.

So what can we say about the mechanism? Well, the original mechanism was going to be ligand binding that goes to increased calcium; intracellular free calcium goes to capping. We would have to say, yes, ligand binding does cause calcium to go up, but you can dissociate these two afterwards. You can get calcium to go up and no capping or you can get capping without any calcium rise. Both stem from the same cause, but they are not sequentially linked. We feel we can accommodate all the old data as well on this hypothesis. If you want to ask why chlorpromazine works, the answer is, it turns out, if you look at it, it kills the ATP inside the cell at the dose they used. Tullio found anything that depletes ATP will stop the whole process. That is no great surprise.

So it is partial confirmation of the old result. But when you can actually measure the free calcium, a rather different answer comes out, when you have the precision to look at the steps before and after the calcium.

That gives me no time to do platelets, so I will skip that.

Why don't I stop there?

DR. ROUX: Questions from the audience?

DR. HEPLER: Are you quite certain that the dye is seeing all the compartments? That is to say, could there be very local compartments, in the case of capping maybe out in the cytoskeletal region, where the calcium does change, but to which the dye, for one reason or another, really is not there, is not seeing this change?

DR. TSIEN: We can't exclude that, of course. When people have looked at these cells under image intensification, fluorescence -- we haven't done that because we don't have the equipment, or we didn't in Cambridge, and I still don't have it in Berkeley yet -- they say it looks uniformly stained. But that is not very high resolution. If you wanted to say that there is a half-micron little patch that had no esterase and so on, or even if it did have esterase, there would be so little dye in it that its signal would be swamped from the nonresponse, yes, that is still possible.

Yes, that is a weakness. If you can get these local domains, then you can postulate that it is not just free calcium, but where it is. That is important.

One reason we have tended to work on small cells, aside from the fact that they are easy to put in a cuvette and suspend, is that we feel that the smaller the cell, the less place calcium has to hide. In a 100-micron cell there are plenty of places to hide. Even in the platelets, which are an extreme case, it is only one micron by three microns. One wouldn't have thought there would be much chance for diffusional barrier, or as much chance.

DR. WATTERSON: I have three questions that are related to that one. They are all related. First, what soluble proteins have you looked for for binding, since it is known that chelators will bind to soluble proteins? Also does the ester group affect the rate and final localization of the molecule? Third, with hydrolysis, do you think there are any changes in terms of pH or whatever?

DR. TSIEN: The first point was, what soluble proteins did we try. The answer is that we considered it rather futile to go through the Sigma catalog ordering all sorts of soluble proteins. What we did is actually loaded quin 2 in the cells and then tried to lyse it by the gentlest possible means that wouldn't let the proteins out. Ideally, we would want holes of the size of about 1,000 molecular weight exclusion. You can try various means of greater sophistication to do that. The one that in the end probably was best was electric shock, a high-voltage electric discharge, which put small holes and let the ions through, but retained lactate dehydrogenase pretty thoroughly. Now, that was the marker we used. Perhaps we should have tested a lot more. We didn't measure calmodulin.

The problem is, that is not perfect. You don't have every cell with exactly the same number of holes. Some cells are still not permeabilized. It is a somewhat random process. Some have bigger holes in them. So you don't get a perfect answer. But insofar as you can tell, the dye comes out very well under conditions in which you can keep in, say, lactate dehydrogenase.

A better way, possibly, in principle, would -- and we didn't push it very hard because the instrumentation wasn't really right for it -- be to measure fluorescence polarization. We can do that without disrupting anything. You figure if it binds to a protein, it will be rotationally immobilized, whereas if it is soluble, it might not be quite as free as aqueous, because there is some gel structure and it is not quite as free. But it will be fairly near free solution values. There was a tendency for a small amount, maybe 50 or 100 micromolar, of the millimolar, typical, to have been a little immobilized, and the rest seemed to be pretty free. But we didn't do it very well. We ought, in principle, to go back and do it, but I am afraid we have gone on to more fun things. This is a control which probably should be done sometime.

Now, the next question was the ester group. We don't see any difference -- not that we have looked systematically. Yes, if the ester group is something that makes the whole ester insoluble, obviously it is not going to work. But within that region, in the limited trial we had with different R groups, which we probably didn't put enough chemical variety in, the cell doesn't seem to care. It usually takes about half-an-hour to an hour in the mammalian system to pretty thoroughly hydrolyze whatever the R. Variations in the acctoxymethyl group have not been explored yet.

Your last one was about acidity, wasn't it? Yes, if you put a lot of the dye on, you get some acidity for a while, because the acetate is being generated. Also, more important, the chelator itself is generating free acid form, and it is impermeable. So it makes four moles of fixed protons. There are four moles of acetate. It seems to us, the few times we did it, that the pH changed by about 0.1 unit acid. It went acid during the loading. It sort of partly recovered, but not completely, by the time we were doing it.

But we have other means for putting acid loads on cells and so on. There are controls you can do by putting in carboxylic acids, which don't chelate calcium, but generate the same byproducts. It doesn't seem to make much difference in the cell's physiology.

But I agree, you should check for toxicity on every new cell prep. It ought to be done. You have to do it on every new prep, because each one might be different.

DR. CORMIER: What is the concentration of the dyes in the cells you have been using?

DR. TSIEN: Inside the cells we tend to use in the range of -- the lowest comfortable readings are at about 0.2 or 0.3 millimolar. The problem is, when you have too little dye, the autofluorescence of the unstained cells begins to dominate. There may be that hint that what dye is in there is a little bit abnormal, because there might be a few saturable binding sites. The highest you want to go is as high as the tissue will permit before it starts getting poisoned, which tends to run 3, 5 millimolar, or as high as you can get. Sometimes the cell esterase isn't active enough to get you much up into that region. So that is our window. A longer wavelength dye you would be able to use a lot less of, because you wouldn't have as much autofluorescence to contend with. It is not the fluorimeter sensitivity that matters at the moment. There is plenty of signal. It is the autofluorescence.

DR. ROUX: Thank you very much.

We will now adjourn for lunch.

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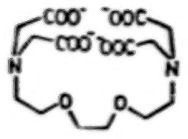
TSIEN FIGURES



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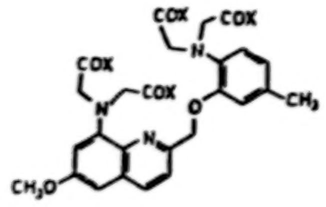

quin2 : $X = O^-$
quin2/AM : $X = OCH_2OCOCH_3$

Fig. 2 (see Ref. 2)

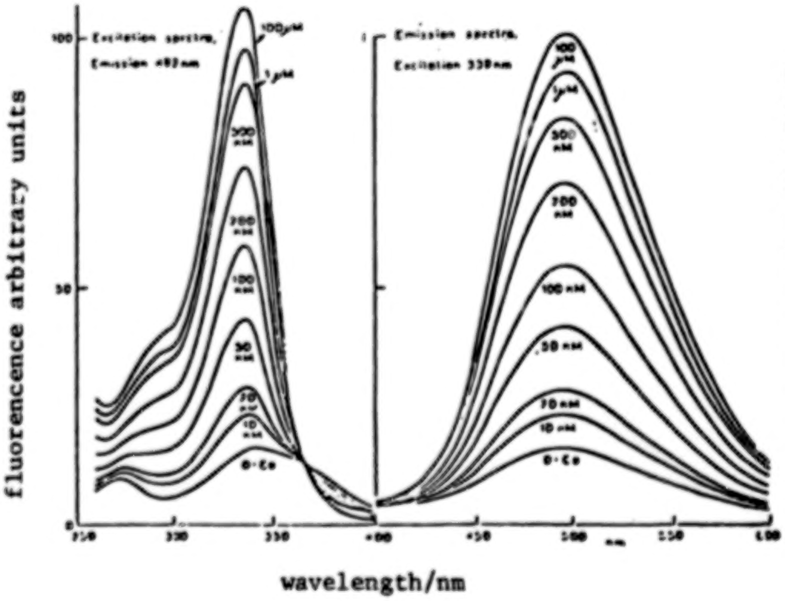

FIGURE 3. Excitation and emission spectra of 20-μM quin2, with varying [Ca^{2+}] as shown against an ionic background of 120–135 mM K^+, 20 mM Na^+, 1 mM free Mg^{2+} and pH 7.05 at 37°C. Further details are given in Materials and Methods. Excitation and emission bandwidths were 4 nm and 10 nm.
(see Ref.2)

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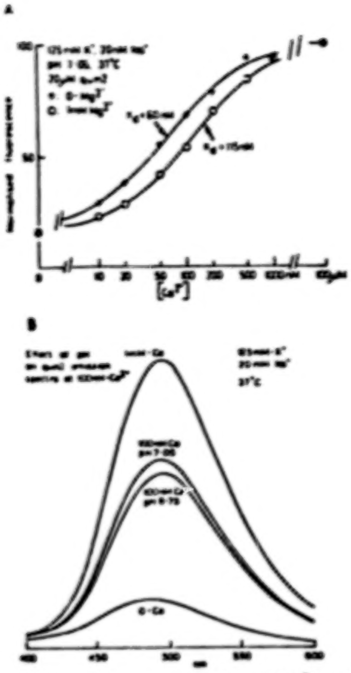

FIGURE 4 Quin2 calibration curves at different $[Mg^{2+}]$ and pH values. (A) Normalized quin2 fluorescence (ex. 339 nm, em. 492 nm) plotted against free $[Ca^{2+}]$ at 0 Mg and 1 mM free Mg^{2+}. The data points are fitted with curves corresponding to one-to-one stoichiometry and the indicated apparent dissociation constants. (B) Shows emission spectra from 20 μM quin2 at "0"-Ca (<1 nM $[Ca^{2+}]$), 100 nM $[Ca^{2+}]$, and 1 mM $[Ca^{2+}]$, at both pH 7.05 and 6.75. The medium contained 125 mM K^+ and 20 mM Na^+. The $[Ca^{2+}]$ and pH were altered by adding appropriate amounts of Ca-EGTA, $CaCl_2$, and K-MOPS while carefully adjusting pH. Changing pH at "0"-Ca and 1 mM $[Ca^{2+}]$ had no effect on the spectra(see Ref.2)

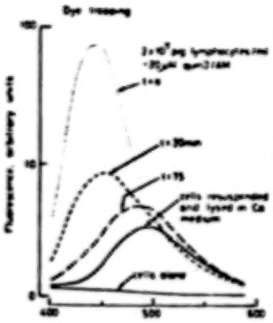

FIGURE 5 Shifts of emission spectra during quin2/AM uptake and hydrolysis. Excitation 339 nm. Dots show the initial fluorescence of external quin2/AM, 20 μM in standard, 1 mM Ca solution. Then 2 × 10^7 pig lymphocytes/ml were added. Dashed curves show the suspension spectra at 20 and 75 min. The solid curve was obtained after washing the cells, resuspending in the same volume of fresh medium, then releasing the trapped dye with digitonin. The bottom trace *cells alone* shows the autofluorescence measured from unloaded cells at the same density.
(see Ref.2)

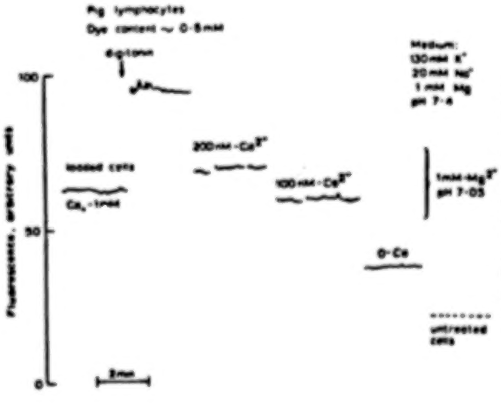

FIGURE 6 Measurement of $[Ca^{2+}]_i$. Fluorescence was recorded from pig lymphocytes containing ~0.5 mM quin2, suspended at 5 × 10^6 cells/ml in medium containing 130 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 5 mM glucose, 10 mM MOPS, pH 7.4. 50 μM digitonin was added to lyse the cells as indicated. $[Ca^{2+}]$ was then set to 200 and 100 nM by appropriate additions of EGTA, with $MgCl_2$ and KOH added to maintain free $[Mg^{2+}]$ at 1 mM and pH at 7.05. The $[Ca^{2+}]$ was set to <1 nM by titration to pH 8.4. The dotted line shows the autofluorescence signal from the same density of unloaded cells. The time bar applies only until the Ca titrations of the medium. Then the periods for addition of EGTA, $MgCl_2$, and KOH are deleted. (see Ref.2)

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TSIEN FIGURES

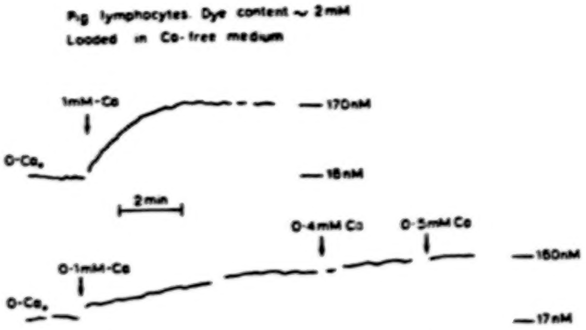

FIGURE 7 Records from cells loaded in low [Ca^{2+}] medium. EGTA was added to the loading medium to reduce [Ca^{2+}]$_o$ below 100 nM. The cells were then washed and resuspended in simplified medium, without added Ca^{2+}. Ca^{2+} was then added back as indicated. The gaps in the traces here, and in subsequent figures, show the times for stirring or addition of reagents. (see Ref.2)

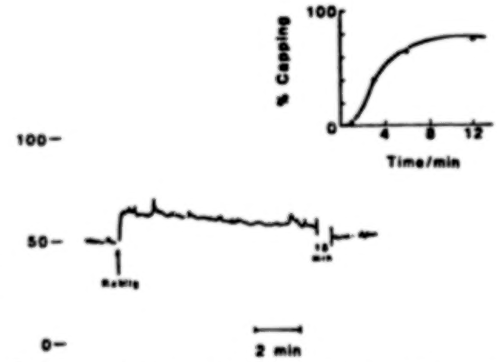

FIGURE 8 Effect of RaMIg on the fluorescence of quin2 trapped in mouse spleen lymphocytes. 10^7 splenocytes per ml, quin2 content ~1 mM, were suspended in 1 mM Ca medium. 53% of the cells were Ig^+, i.e. could be stained with FITC RaMIg. 40 µg/ml unlabeled RaMIg was added when indicated. The occasional brief random spikes on the fluorescence record are due to fluctuations of lamp intensity or small bubbles intersecting the beam, and should be ignored. The calibration marks for 0 and 100% Ca-saturation correspond to F_{min} and F_{max} as defined in the previous paper (14), and denote the fluorescence levels attained when the intracellular quin2 was released by Triton X-100, exposed to <1 nM and 1 mM [Ca^{2+}], respectively, and the resulting limiting fluorescences corrected slightly for cell autofluorescence. The inset shows cap formation as a function of time in the same batch of cells exposed to the same concentration of RaMIg. (see Ref.5)

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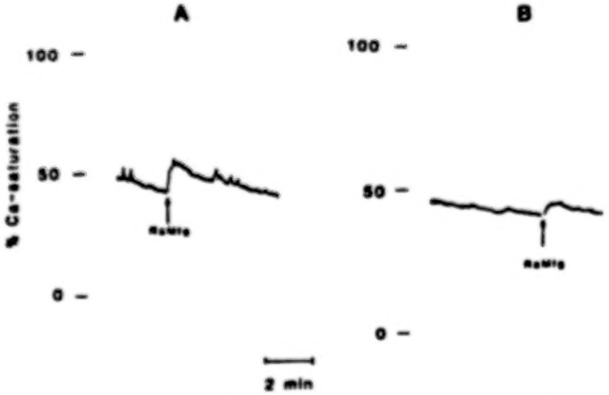

FIGURE 9 Responses of quin2 fluorescence to anti-Ig in Ca-free solution (zero added Ca, 200 μM EGTA) at different quin2 loadings. 40 μg/ml RaMIg added as indicated. Cells 50% Ig^+. (*A*) Quin2 content, 1 mM. (*B*) Quin2 content, 3 mM. (see Ref.5)

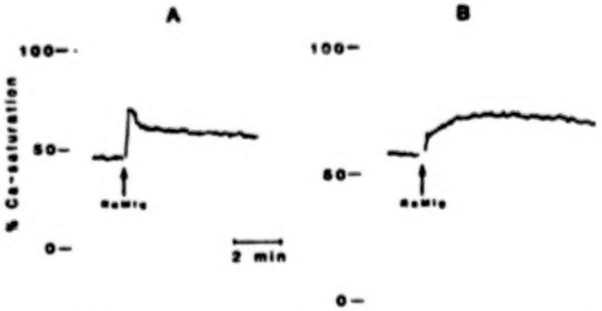

FIGURE 10 Responses of quin2 fluorescence to anti-Ig in 1 mM Ca solution at different quin2 loadings. 40 μg/ml RaMIg added as indicated. Cells 58% Ig^+. (*A*) Quin2 content, 0.7 mM. (*B*) Quin2 content, 3 mM. (see Ref.5)

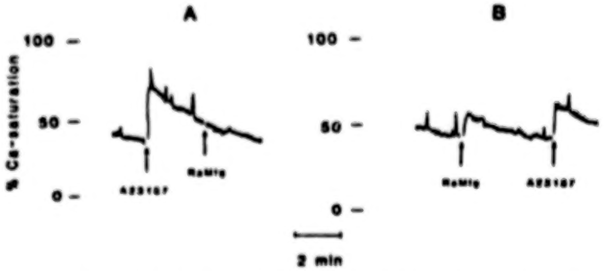

FIGURE 11 Effects of A23187 on $[Ca^{2+}]_i$ in Ca-free medium. Quin2 content, 1.4 mM, 44% Ig^+. Addition of 40 nM A23187 and 40 μg/ml RaMIg, as indicated. For unknown reasons the noise "glitches" were unusually severe in this experiment. (see Ref.5)

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DR. ROUX: The first speaker this afternoon will be Dr. James Chafouleas from Baylor College of Medicine, and his title is "Role of Calmodulin in Cell Proliferation."

DR. CHAFOULEAS: The studies I will be talking to you about today were done in collaboration with Tony Means at Baylor. About five years ago, we decided that most labs were really looking at the mechanism by which calmodulin regulated its target proteins, the enzymes it was interacting with. Since the regulation mediated by calmodulin is ultimately dependent on the regulation of calmodulin itself, we turned our attention towards evaluating how calmodulin is regulated in cells.

We looked for an experimental system where calmodulin could be altered. We first looked to see whether hormones had any effect on the calmodulin levels in their respective target cells.

In collaborative studies with Mike Conn at Duke (1) we found, to our initial dismay, that calmodulin levels were not under any apparent hormonal regulation but were constitutively expressed in target cells for both steroid and peptide hormones. However, calmodulin was indeed affected by hormone action on the target cell, in that peptide hormones that regulate secretion promote the intracellular redistribution of calmodulin. This redistribution occurred from cytoplasm to plasma membrane and was coincident with secretion. Termination of hormone stimulation was accompanied by the reestablishment of the calmodulin distribution evident in the nonstimulated cells.

So at least with respect to hormone action, the total levels of calmodulin in the cell don't change, but local elevations and decreases within the cell occur.

While calmodulin levels were not altered by hormones, Watterson et al, and La Porte et all, had reported that calmodulin levels might be elevated in transformed cells. Since these studies quantitated calmodulin levels by bioassay or densitometry, we decided to use our radioimmunoassay to confirm this and determine the mechanism by which this elevation occured.

These studies were done in collaboration with Bill Brinkley, John Dedman and Bob Pardue at Baylor and are summarized in Ref. (2). We found that indeed transformation of cells by oncogenic viruses, chemical carcinogens, and hormones resulted in at least a two- to threefold elevation in calmodulin levels relative to the nontransformed counterparts. This elevation was due to an increase in the rate of synthesis, and not degradation, of calmodulin, and that this effect was selective for calmodulin, since neither tubulin nor actin content was altered. Finally, given the multifunctional aspect of calmodulin, we proposed that the elevated calmodulin level may account for some of the phenotypic changes characteristic of the transformed cells.

These studies (3) were performed on asynchronous populations of cells in culture, and we were interested in finding out where during the cell cycle this calmodulin synthesis occurred. We turned our attention to the Chinese hampster ovary (CHO) cell for these studies, for many reasons. They have a very short cell cycle time relative to a lot of other mammmalian tissue culture cells. They are easy to culture and most importantly can be synchronized without the use of drugs in mitosis, by a procedure called the mitotic shake procedure.

In the mitotic shake procedure the rounded-up mitotic cells are shaken free from the surface of the flasks and are collected into medium. The cells are maintained in mitosis by simply placing them into medium at four degrees. The cells are released from this mitotic block by placing them into medium at 37 degrees, at which time they will synchronously exit mitosis and progress into and through G_1.

At the top of Figure 1, I have superimposed the different lengths of the various phases of the cell cycle. At time zero, the cells were released from the mitotic block and allowed to progress into G_1. As you can see in the lower panel, the starting M population of cells have a mitotic index of approximately 98 percent. Routinely in these experiments, the mitotic index was greater than 95 percent. One hour after release into the medium at 37 degrees, the cells had exited mitosis and entered into G_1, resulting in a mitotic index of zero. This level was maintained for the next eight to ten hours, after which we start to see the repopulation of mitotic cells, demonstrating that the cells have traversed the cell cycle and are getting back into mitosis.

Progression into S phase was monitored by tritiated thymidine pulses at various time points as shown in the upper panel. The starting M population of cells has a zero labeling index, which remains constant throughout the early part of G_1. Approximately three to four hours after release, the cells synchronously enter S phase and undergo DNA synthesis, reaching a peak labeling index approximately eight to ten hours after release. The cells then start to exit S phase and enter G_2, as demonstrated by the reduction in the labeling index.

When we looked at calmodulin content using the radioimmunoassay, we found that the starting M population of cells had approximately 155 nanograms for 10^6 cells. One hour after release from mitosis, this value had been reduced by 50 percent, as you would expect, since each mitotic cell now had undergone cytokinesis, resulting in two G_1 cells for every starting mitotic cell. This intracellular value for calmodulin is maintained for the next three to four hours, at which time there is a rapid increase in the calmodulin content, which is coincident with the progression of the cells into S phase. The calmodulin concentration reaches the 155 nanograms per 10^6 cells observed the starting M population of cells, and is maintained throughout the rest of the cell cycle.

These data suggest that the calmodulin was synthesized at the G_1/S transition. We asked the question whether the length of G_1 had any effect on the positioning of the calmodulin synthesis. One major advantage of the CHO cell is that we can grow the cells in a different serum-containing medium, and alter their doubling time through a change in the length of G_1. So we changed the serum milieu of the medium and obtained a CHO cell population with a 12.5 doubling time that was solely a result of the reduction in the G_1 phase from 5 hours to 2 hours. We then looked at the calmodulin levels in these synchronized cells.

In Figure 2, I have simply replotted the calmodulin values and the labeling index from the past slide for comparison purposes, focusing on the first eight hours of the experiment. When we looked at the calmodulin values for the CHO cells which had a shorter G_1 phase of 2 hours, we found that the starting M population of cells also had 155 nanograms per 10^6 cells. Again, after they have exited mitosis and entered G_1 phase, this value was reduced by 50 percent. In contrast to the longer G_1 phase cells, this value is only maintained for another hour, after which there is a rapid increase in this value back to the 155 nanograms per 10^6 cells observed in the starting M population of cells. Again, this is maintained throughout the duration of the cell cycle. You will note that the cells do indeed have a shorter G_1 phase and that the increase in the calmodulin concentration is again coincident with or slightly preceding the progression of the cells into S phase. These data were very suggestive that the levels of calmodulin may be important for the ability of the cells to get into S phase and undergo DNA synthesis. We performed four independent experiments using the CHO cells with the five-hour and the two-hour G_1 phases, and compared the relative calmodulin concentration to the relative labeling index. As you can see in Figure 3, there is a very positive correlation between the calmodulin concentration and the labeling index, suggesting that calmodulin may be important or involved in the ability of the cells to go through the G_1/S transition of the cell cycle.

If that were the case, we reasoned that we should be able to inhibit this progression using some of the anticalmodulin drugs that were available. As Milt Cormier told you yesterday, a great deal of caution has to be used in any studies using anti-calmodulin drugs. We chose to use the drugs of the naphthalenesulfonamide class that he spoke to. We didn't use the W5, W7 drugs that he spoke to you about yesterday; we used another group of drugs called W12 and W13.

As you can see from the structures of W12 and W13, both drugs are identical except for the addition of a chlorine on the W13 molecule (Table 1). Apparently, this chlorine molecule imparts a higher affinity for calmodulin to the W13 molecule, as evidenced by its ability to displace the other anti-calmodulin drug Milt talked to you about yesterday, W7. W13 has a fivefold increase in affinity for calmodulin, and this fivefold increase in affinity is expressed as approximately a fivefold increase in efficacy to inhibit two biological activities of calmodulin, activation of cyclic nucleotide phosphodiesterase and myosin light-chain kinase.

Although they show different affinities and efficacies of inhibition, both drugs have very similar hydrophobic indices, and so the use of W12 as a control drug should alleviate some of the problems found in the studies using the phenothiazines.

The first order was to determine the cytotoxicity of these drugs for the cultures. The experiments were carried out by treating the CHO cells, in exponential growth, for 24 hours in various concentrations of either W12 or W13, and then determining cell survival by colony formation. As you can see in Figure 4, W12, which is the closed triangles, essentially has no effect on cell survival up to concentrations of 80 micrograms per ml, and then above that concentration there is only a slight increase in cytotoxicity, with approximately 60 percent cell survival at a dose of 150 micrograms per ml. W13, on the other hand, shows a markedly different profile, in that, while there is no cytotoxicity at 30 micrograms per ml of W13, increasing that concentration just to 35 micrograms per ml results in 40 percent cell kill. If we go to a dose of just 60 micrograms per ml, we have essentially killed all the cells in the culture.

We therefore decided to see what effect the noncytotoxic dose of 30 ug/ml of W13 or W12 would have on cell proliferation. This study is shown in Table 2.

For this experiment, one million cells per plate were seeded at time zero into either fresh medium or medium containing 30 micrograms per ml of W12 or 30 micrograms per ml of W13. Eight hours later, replicate plates were removed and cell counts were determined. As you can see, after eight hours of exposure to the drugs, there is essentially no difference in the resulting cell numbers between any of the three groups.

If we then looked at cell counts in cultures 24 hours later, we found that there was a nice increase in the cell number for the controls and that there was essentially no difference from the control values for W12-treated cultures. However, now we found a 52 percent decrease in the resulting cell numbers for the W13-treated cultures. These data suggested that W13 was having an effect on the ability of the cells to proliferate. We then asked the question, where during the cell cycle was W13 imparting this inhibition?

We first looked at the ability of the cells to go through G_1 into S phase. For these experiments, cells were again synchronized in M phase by the mitotic shake procedure and then released at zero hour into normal medium, at 37 degrees. I should mention now, that all the experiments I will be showing you for the rest of the talk deal with CHO cells with the two-hour G-1 phase. One hour after release from mitosis, we either treated the cultures with fresh medium or fresh medium containing W12 or W13 at 30 micrograms per ml, and then monitored progression into S phase with tritiated thymidine pulses.

As you can see in Figure 5, both the control and the W12-treated cultures enter S phase approximately two hours after release, reach a peak labeling index about seven or eight hours after release, then start to progress through S phase into G_2. Cultures which were treated with W13, on the other hand, show a marked diminution in the population of cells that are capable of getting into S phase.

When we looked at the effect of the drugs on the ability of cells to progress through S phase, we found the following. For this experiment, cells were synchronized at the G_1/S boundary by the double thymidine procedure, and at time zero were released from the high-thymidine block into fresh medium. One hour after release, the cultures were either treated with regular medium or medium containing the two drugs, W12 at 30 micrograms per ml or W13 at 30 micrograms per ml, and then progression was monitored by tritiated thymidine pulses.

As you see in Figure 6, at one hour after release from the double thymidine block, the cells are indeed in S phase, as evidenced by the high labeling index. Control and W12-treated cultures progress nicely through S phase. The cultures start to exit S phase and enter into G_2, as demonstrated by the reduction in the labeling index, until at seven hours we have a labeling index for control and W12 treated culture of approximately 38 percent.

Cultures which were placed in W13, on the other hand, appear incapable of progressing through S phase, as you can see by the relatively flat profile of the labeling index. At seven hours after release, the labeling index has only dropped to 76 percent, suggesting that there was a marked effect on the ability of the cells to progress through S phase.

SPEAKER: Does that mean they are still synthesizing DNA?

DR. CHAFOULEAS: They are still taking up the thymidine, and incorporating it into DNA. They are not progressing through S phase.

DR. BIKLE: I am having trouble understanding why that means that they are arrested in S phase, as opposed to going all the way around and arresting at mitosis.

DR. CHAFOULEAS: Well, they don't incorporate thymidine into DNA in mitosis. These are tritiated thymidine pulses. If you remember, S phase is where DNA is synthesized. All right. We next looked at the effect of the drug on G_2 progression. That is shown in the next slide. As you can see in Figure 7, there is essentially no effect of W13 on the ability of the cells to go through G_2 phase. As shown in Figure 8, there is no effect on the ability of the cells to progress through mitosis.

This observation was relatively surprising to us, given the fact that one of the earliest observations on the intracellular localization of calmodulin, as Peter Hepler pointed out, was that it decorated the half-spindle of the mitotic apparatus. We felt that, of any of the phases of the cell cycle, this was the phase where the drug should have had an effect. However, at the sublethal dosage that we are using, the only sensitive portions of the cell cycle appear to be at the G_1/S transition and S phase, and not mitosis.

In summary, we found that calmodulin synthesis occurs at the G_1 boundary. Calmodulin levels increase twofold at the G_1 and remain elevated until cytokinesis, after which time cell division results in a 50 percent decrease in the calmodulin concentration back. The calmodulin increase is tightly coupled to entry into S phase, and the anti-calmodulin drug specifically and reversibly prevents progression through S phase.

Now, what is very nice about the positioning of the calmodulin synthesis at G_1/S is that that is a very sensitive position of the cell cycle, as I am sure most of you are aware. It is also during G_1 phase that the decision is made to either exit the cell cycle and go into a quiescent phase or continue through cell replication. We wanted to ask the question whether calmodulin was somehow involved in the progression of cells into and out of plateau (G_0) phase. Again, the CHO cells are ideally suited for this, in that they can be induced to go into a quiescent phase by simply growing them to a high density. That is shown in Figure 9.

As shown in the upper panel of Figure 9, if we plate CHO cells in culture dishes at 5×10^5 cells, there will be an exponential growth for the first 60 hours. Then when they reach a critical density, usually at 1×10^7 cells, they cease cell division, the cell number plateaus, and the DNA synthesis decreases so that the labeling index falls below three percent in this population of cells. Now, although cell division ceases and the cells are very quiescent, they are indeed viable, and you can subsequently release them from this plateau phase by simply replating them at a lower density into fresh medium. There will be a subsequent lag period, after which they will be able to go through DNA synthesis and divide again. Cultures can be maintained in plateau phase for up to 60-80 hours and routinely demonstrate a plating efficiency of at least 93%.

When we looked at the calmodulin levels during the course of this experiment, we found the cultures had approximately 128 nanograms per 10^6 cells throughout exponential growth. When the cells began to enter into plateau phase, this concentration increased slightly but significantly, to approximately 150 nanograms per 10^6 cells, and then maintained that level throughout plateau phase.

Now, as I said before, we can release the cells from plateau and induce them to go back into the cell cycle. If we did that and looked at the calmodulin levels, we found the following (Figure 9). Again, the starting plateau phase cells had 155 nanograms per 10^6 cells. Within one hour after release, this value was reduced by 40 percent. This is a very consistent observation. This value then is maintained for the next four hours, after which there is a marked increase in calmodulin to the 155 nanograms per 10^6 cells that we saw in both the normal M cells of the cell cycle experiments and the starting plateau phase cells. This value is maintained throughout the duration of the cell cycle, and follows the same pattern you saw before. In addition, you will note for the control cultures are entering into S phase at approximately six to eight hours after release from the plateau phase.

These two changes in calmodulin levels were very interesting and suggested that calmodulin may be involved in the ability of cells to exit the plateau phase and enter into the cell cycle. If that were the case, then we should be able to block the cells from getting into the cell cycle by W13. That is shown in Figure 10.

Cells were in plateau phase, and at zero hour were released from plateau by simply resuspension at a lower density into fresh medium. Progression into and through S phase was monitored as before using tritiated thymidine. If the cultures were resuspended into either fresh medium or medium that contained 30 micrograms per ml of W12, we found that, in about six to eight hours, they started to enter into S phase and reached a peak labeling index approximately 16 to 18 hours after plateau release, and then progressed through S phase and into G_2 phase. You will note, however, if the cells were plated into medium that contained 30 micrograms per ml of W13, there was absolutely no progression into S phase throughout the duration of the experiment, up to 24 hours after release from plateau phase. Control cells and W12-treated cells were indeed cycling through the cell cycle, since we started to see an increase in the cell number 20 to 24 hours after release from plateau phase. Again, as you would expect from these data here, cultures which were treated with W13 show no increase in cell number throughout the duration of the experiment.

This was very interesting, and we decided to find out where during plateau reentry W13 was imparting its inhibition. This is also shown in Figure 10. For these experiments, cells again were in plateau phase, and at zero hour released from plateau phase. They were either released into fresh medium or medium containing W13 at various times after plateau release. The different times of drug treatment after plateau release are written, next to their respective labeling indices. The control and W12-treated cultures synchronously progress into S phase six to eight hours after release, reaching a peak labeling index at 16 to 18 hours. These cultures then subsequently progress out of S phase into G_2 phase and undergo cell division. Cultures which were plated directly into W13-containing medium failed to progress into S phase. You will note, however, if we initially plated the cultures into fresh medium and then, at various times, one, three, or five hours later, added W13 at 30 micrograms per ml, we saw successively greater labeling indices. However, although the cells were getting into S phase, they appeared to be incapable of progressing through S phase, as demonstrated by the plateau in the labeling index. If the cultures were treated with W13 nine or twelve hours later, we again see successively greater labeling indices, as well as a group of cells which are capable of getting out of S phase.

We next asked the question whether the effect of W13 on the inhibition of plateau release was reversible. This is shown in Figure 11. Focus your attention to panel A. For these experiments, again, the cultures were in plateau, and at time zero were released from plateau by resuspension at a lower density. For panel A, the cultures were resuspended into medium that contained W13 at 30 micrograms per ml. As we have seen before, the cultures are incapable of progressing into S phase as long as the drug is present in the medium. If we remove the drug from the medium 18 hours after plateau release, or a total exposure time of 18 hours of the drug, we see that there is approximately an eight- to ten-hour lag period, which is then followed by a synchronous progression of cells into S phase, as evidenced by the labeling index.

As shown in Panel B, cultures treated 5 hours after plateau release are moderately capable of entering into S phase reaching a peak labeling index of 36%. When the drug was removed 18 hours after plateau release, or a total drug exposure time of 13 hours, we found that after a four-hour lag, those cells that were in S phase began to progress out of S phase. This was followed 4 to 6 hours later by the synchronous progression of another group of cells into S phase. You will note that this profile for the second group of cells is relatively similar to the profile observed when the drug was added at zero time.

In Panel C the same experiment was performed as in Panel B, except that the drug was kept on the cultures for an additional five hours. You will note that, no progression through S phase is observed for the additional five hours of drug treatment and that, as with Panel B, within four hours after drug removal, the cells that are in S phase progress out of S phase, followed by another population of cells which synchronously progress into S phase. This suggests that we have at least two places where W13 blocks the cells in plateau release, one very early, possibly right at the beginning, and one that seems to be coincident with a very early S phase block, something very similar to what we have seen in normal cell cycle progression.

In summary, we have found that calmodulin levels increase as cells enter plateau. The data suggest that the cells are exiting the cell cycle late in G_1 phase, or that the calmodulin levels in plateau phase cells are uncoupled to progression into S phase in plateau cells. Upon release, calmodulin levels rapidly decrease. We are currently looking into the possible mechanisms by which this could occur. Following this decrease there is an increase prior to S phase, and we feel that that increase is probably very similar to the increase we saw in the normal cycling cells, which is a prerequisite for the ability of the cells to get into S phase. The anti-calmodulin drugs inhibit progression into S phase, this inhibition is reversible and is correlated with the time of drug addition following plateau release.

Finally, we see the involvement of calmodulin in cell proliferation as follows. Following mitosis, cytokinesis results in a reduction in the calmodulin levels, to a 1-X value. This value is maintained until the cells have made the commitment to enter into S phase. Following this commitment there is a period in which a transient change in calmodulin level occurs, until it reaches a 2-X value. This value is then maintained throughout the duration of the cell cycle.

If we look at the calmodulin levels during plateau reentry, we observe that the plateau phase cells contain the 2-X value of calmodulin. Release from plateau phase is associated with an immediate reduction in the intracellular calmodulin level which is maintained until just prior to entry into S phase at which time the calmodulin levels increase to the 2-X value.

You will also note that drug sensitivity is where the calmodulin levels are changing in the cell cycle.

We are now directing our attention to determining the mechanisms by which the calmodulin levels are changing, whether the regulation is a transcriptional or translational event. Hopefully we will have some new information about that.

Thank you.

DR. ROUX: Any questions?

DR. WATTERSON: I have a few questions. Do you know that the naphthalene sulfonic acid is getting into the cytoplasm?

DR. CHAFOULEAS: Yes, within five minutes.

DR. WATTERSON: Secondly, what is the effect of W12 and W13 on dissociating free-form calmodulin in enzyme complexes? In other words, how rapidly does that occur? Most of the hypotheses of the mechanism of action are that it blocks formation of unformed complexes. There is plenty of evidence, in biochemical aspects, that there are free formed complexes around. Will it dissociate those? And if so, is the time course of that within the window of the cell cycle?

DR. CHAFOULEAS: Well, I haven't really looked at that. I am sure that Hidaka has. I am not sure how long it takes for it to inhibit the PDE or the myosin light-chain kinase assay, so I can't give you a time course on that. But obviously it must have some effect on the ability of calmodulin to interact with the PDE. I do know that Hidaka has added the drug both before and after adding the calmodulin to activate the phosphodiesterase, and he gets the same net result. But I didn't do those experiments, and so I don't know exactly how they were performed.

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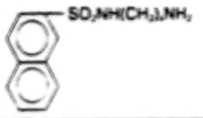
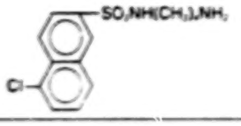
CHAFOULEAS TABLES

Table 1. Structure and Anticalmodulin Activity of W12 and W13

W12



W13



	IC_{50} (μM)[a] W12	IC_{50} (μM)[a] W13
Displacement of ^{3}H-W7 from calmodulin[b]	280 ± 30	55 ± 5
Inhibition of phosphodiesterase activation[c]	260 ± 40	68 ± 4
Inhibition of MLCK[d]	300 ± 35	58 ± 6

W12: N-(4-aminobutyl)-2-naphthalenesulfonamide.

W13: N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.

[a] The IC_{50} is defined as the concentration of drug required to displace 50% of the labeled W7 from calmodulin or to produce 50% inhibition of each enzyme activity. All experiments were performed in triplicate and data are presented as the means ± SE.

[b] Displacement of ^{3}H-W7 was determined by the equilibrium binding technique of Hummel and Dryer (1962), with Sephadex G-50 as previously reported (Hidaka et al., 1980b).

[c] Calmodulin-dependent cyclic nucleotide phosphodiesterase was purified from bovine brain and assayed under standard conditions previously described (Hidaka and Asano, 1976).

[d] Myosin light chain kinase (MLCK) was purified from chicken gizzard, and enzyme activity was measured as previously described (Hidaka et al., 1980b).

Table 2. Effect of W12 and W13 on Cell Number

	Cell Number (× 10^{-5})			
Treatment	0 hr	8 hr	24 hr	% Control
None	1	1.98	4.95	100
W12	1	1.60	5.00	101
W13	1	1.89	2.38	48

Cells in exponential growth in 60 mm dishes were treated with medium alone or medium containing W12 or W13 at a final concentration of 30 μg/ml at time zero. Total cell counts were performed at 8 and 24 hr as described in Experimental Procedures. The data presented in this table are from a single experiment. The experiment was repeated four times. No significant interassay or intra-essay differences in cell number were noted between any treatment group at 8 hr. In each case, the number of cells present after 24 hr in the nontreated group was set at 100%. In the four experiments the values for W12 treatment were 100% ± 3%, whereas the values for W13 treatment were 47% ± 2%.

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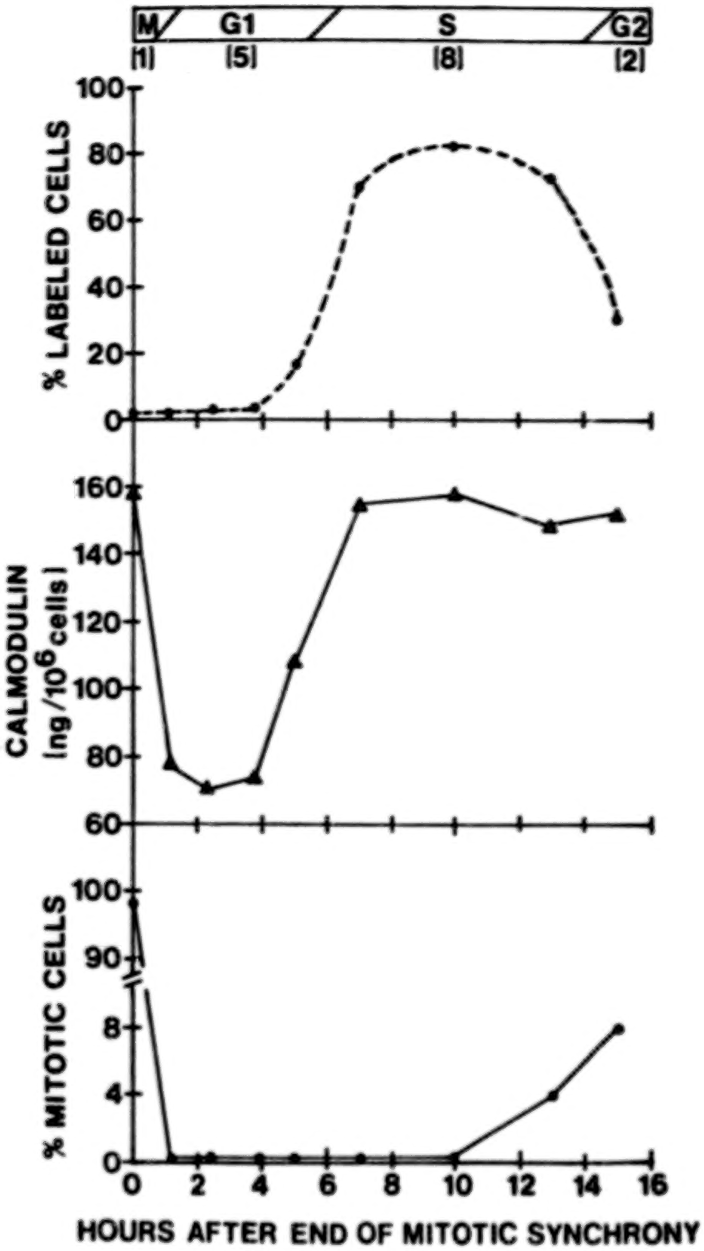

FIGURE 1

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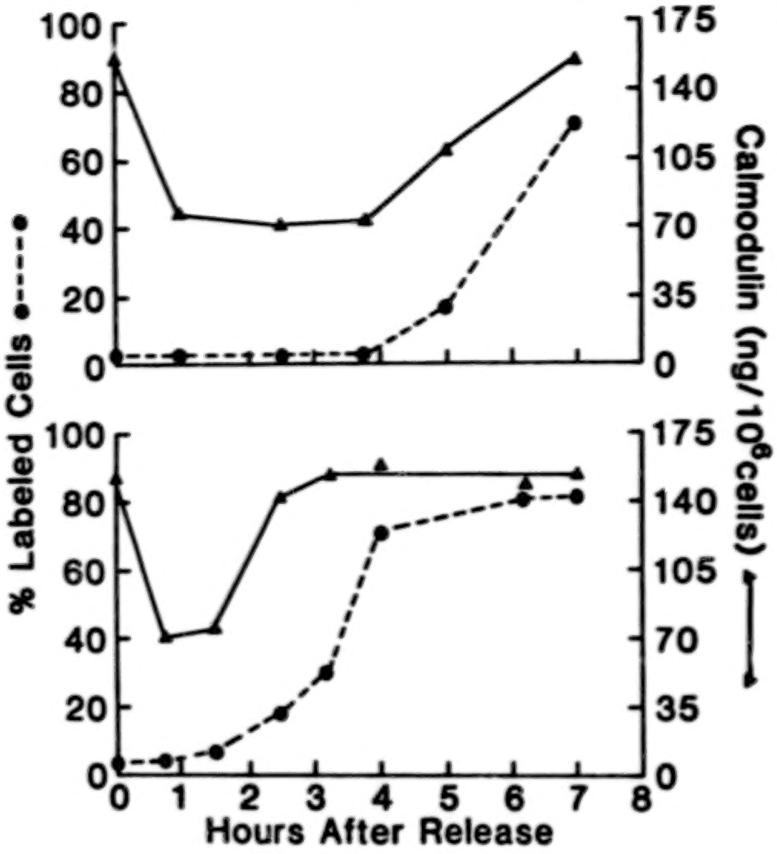

FIGURE 2

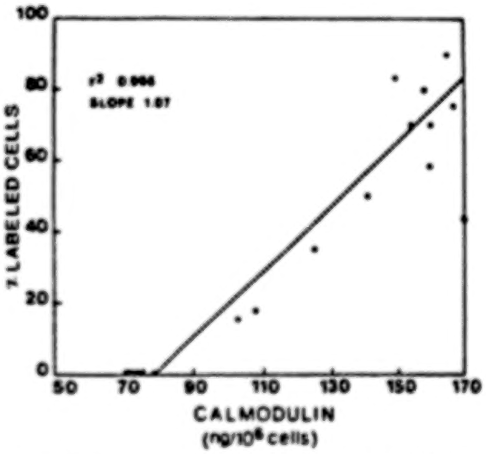

Figure 3. Correlation between the Intracellular Calmodulin Levels and Progression of Cells into S Phase

The ^{3}H-thymidine labeling index is plotted against the appropriate intracellular calmodulin concentration from four independent experiments with CHO-K1 cells with G1 periods of 5 and 2 hr as described in Experimental Procedures.

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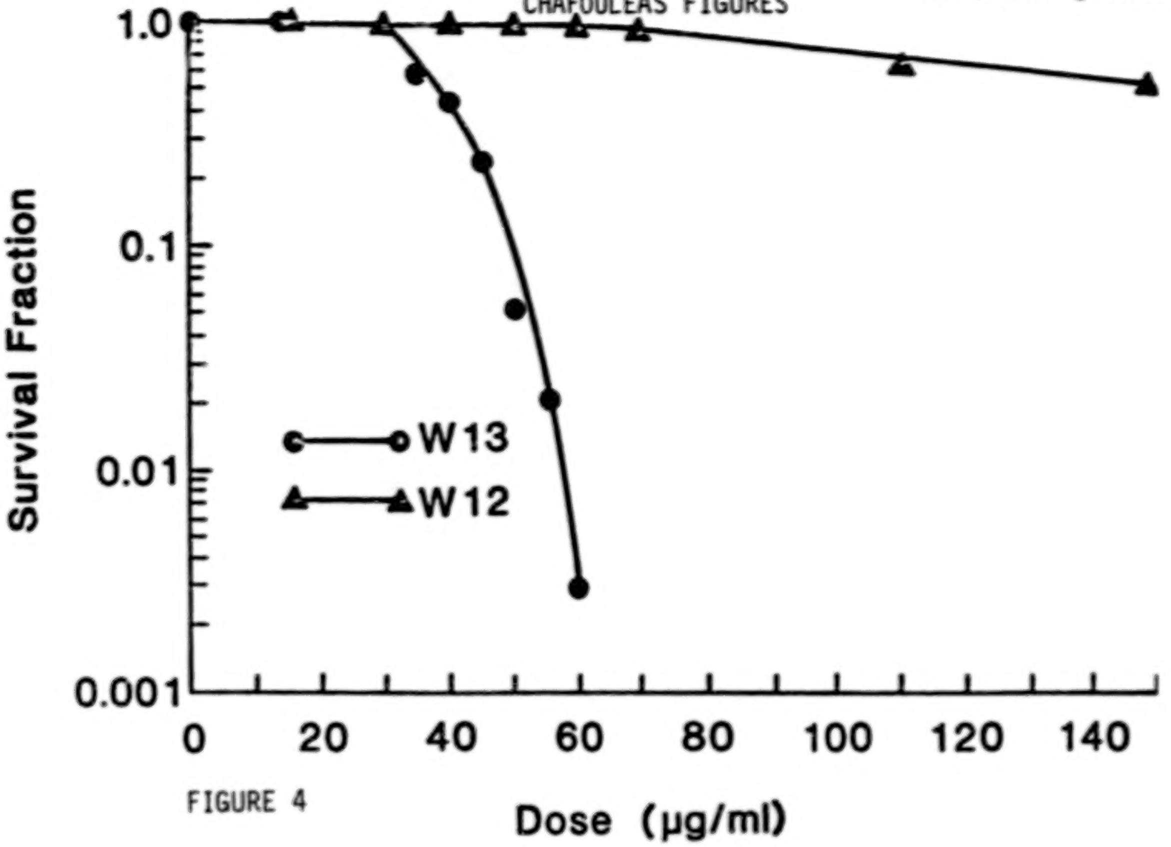

FIGURE 4

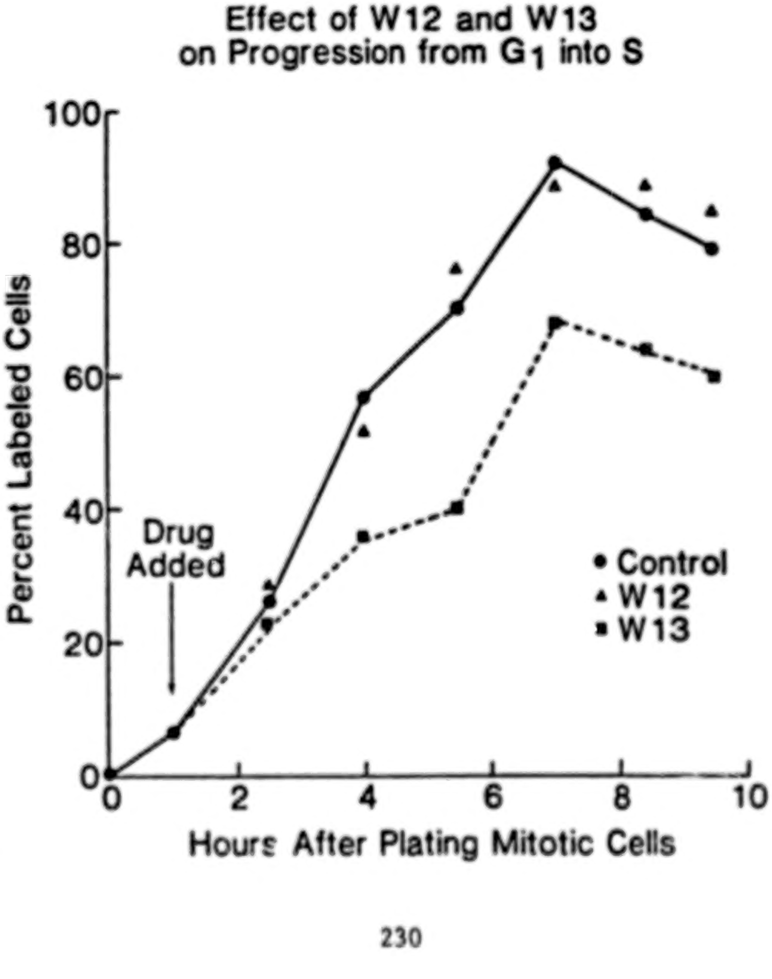

FIGURE 5

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Effect of W12 and W13 on S Phase Progression

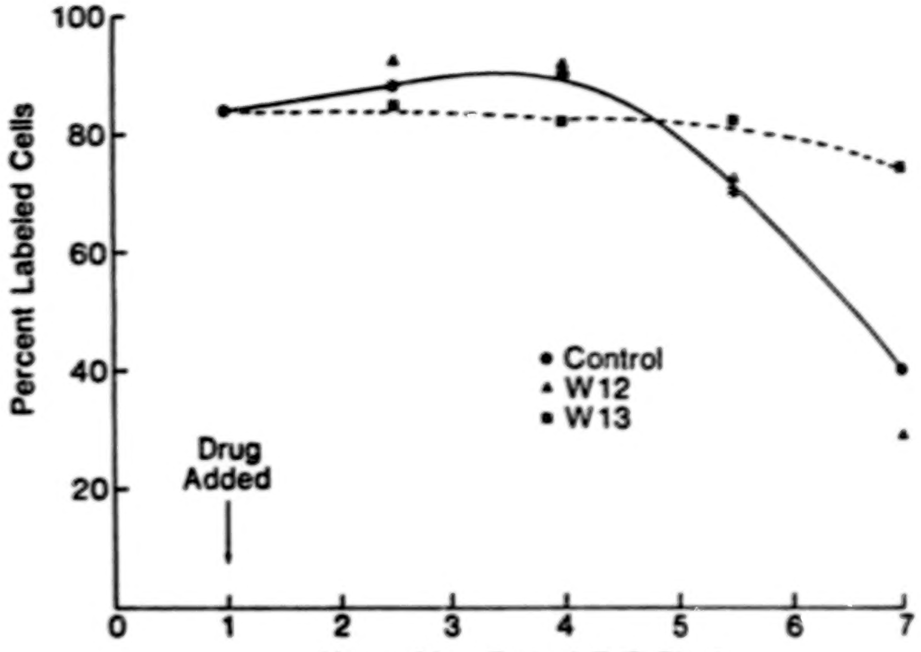

FIGURE 6

Effect of W12 and W13 on G_2 Progression

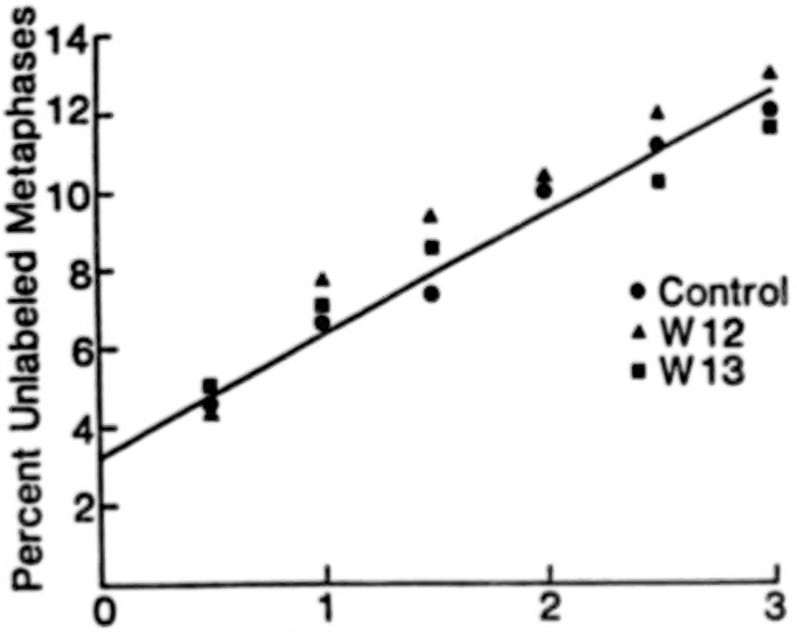

FIGURE 7

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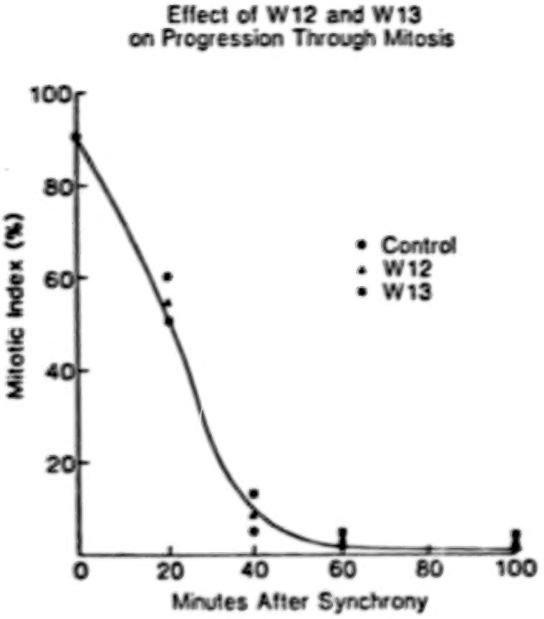


Effect of W12 and W13 on Progression Through Mitosis

FIGURE 8

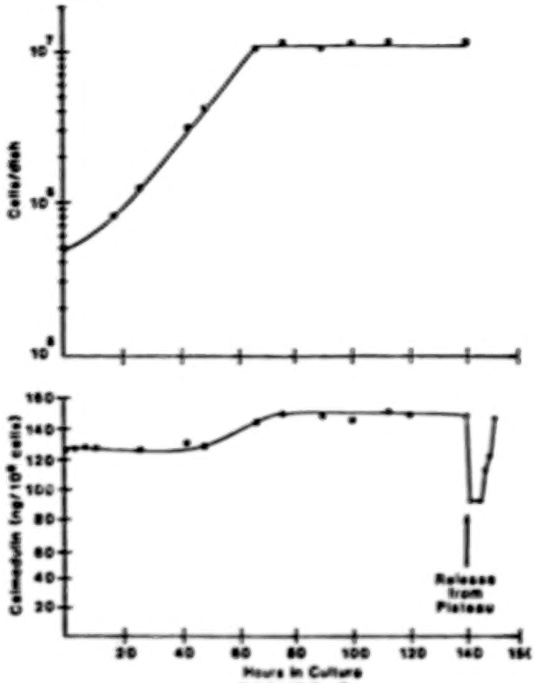


FIGURE 9

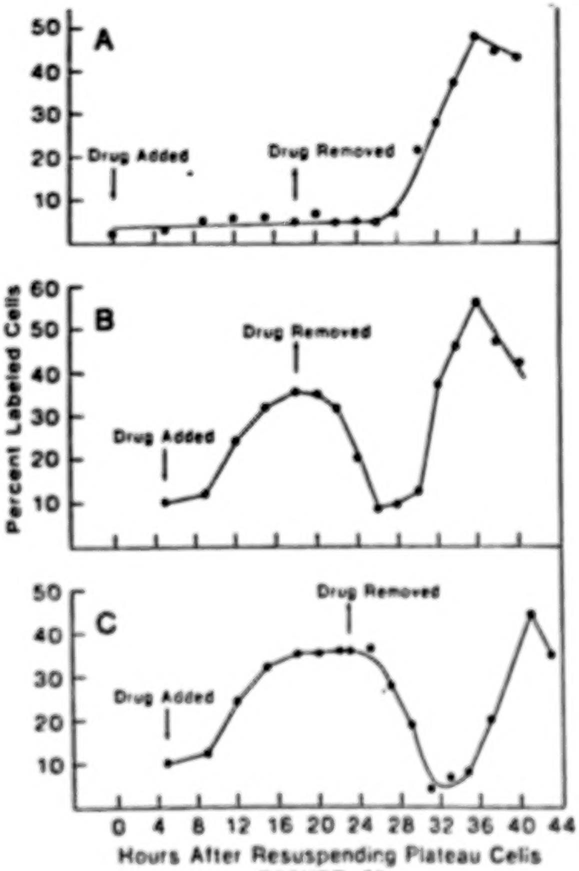
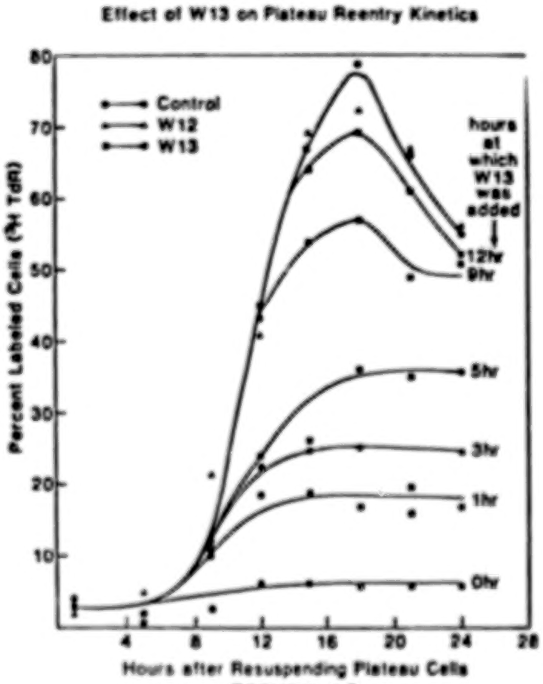


FIGURE 11



Effect of W13 on Plateau Reentry Kinetics

FIGURE 10

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DR. ROUX: Thank you very much. The next speaker in this afternoon's session is Dr. Michael Bennett from the Albert Einstein College of Medicine. Dr. Bennett will be talking to us about "Controls of Intracellular Communication Mediated by Gap Junctions."

DR. BENNETT: Thank you. Figure 1 is a cartoon, resulting from a lot of work by many people, but the drawing is Don Casper's and Dan Goodenough's. It is a diagram of the gap junction, showing phospholipid moieties in the membrane, the intercellular space, which is the gap of the gap junction, and the intercellular channels, several shown in longitudinal section. Each channel is about 12 to 14 angstrom in diameter.

The individual channels, or connexons in Goodenough's term, are shown as made up of a hexamer in each membrane of the protein that can be isolated from the junctions.

Where does this picture come from? Well, to briefly summarize that literature (1): Electron micrographs of thin sections of gap junctions show two membranes of joined cells to be closely opposed and quite parallel, with a little electronlucent gap in between them, which is the gap of the gap junction. Extracellular space markers can get into the gap.

Now, from Figure 1, it is clear that gap junction is a bad name in terms of describing the function of this structure. It should be called a communicating junction or a nexus. Both terms are occasionally used, but gap junction is very deeply embedded in the literature now, and we are probably stuck with it.

One can isolate gap junctions by using detergent and end up with quite pure preparations which in thin section look like in situ gap junctions without any of the other cytoplasmic muck associated with them. In negative stain of such isolated junctions, one sees hexagonal crystalline arrays of the intercytoplasmic channels. This material has been subjected to analysis for protein. There is some disagreement as to how big the protein is, but the major constituent has a molecular weight of about 26,000. That is about right for the channel to be made up of a hexamer of those particles within each membrane.

The techniques of morphological investigation of the gap junction are being pushed forward, or downward, to better and better resolution. While we aren't entirely there yet, the picture is getting to look rather more like the diagram that we started out with at the beginning.

In terms of physiology, the gap junction mediates electrical coupling between cells. One can take pairs of cells, put electrodes in them, pass current in one cell which produces a big potential in that cell and a smaller potential in the other cell, and then pass current in the other cell which produces a larger potential in that cell and a smaller potential in the first cell. This is the electrical coupling. The gap junction is also called an electrotonic junction, or electrotonic synapse, when it is between neurons.

One knows that there is a special relation between these neurons. It is not coupling by way of extracellular space, as one can show by calculations and by moving electrodes around and looking in different regions outside the cell and inside the cell. There is a specialization that connects them, and the only place where they are close together is at gap junctions. When one has gap junctions, one has coupling, and when one doesn't have gap junctions, one doesn't have coupling, at least of this kind.

The gap junctions also permit the passage of small molecules between the cells, which is where the evidence for the diameter of the channel comes from. The pathway is, after a lot of investigation, nonspecific. It transmits almost any molecule in diameter up to about 12 angstroms.

There were a lot of data obtained, looking at different neurons and in different tissues, which indicated that there are simple channels between cells with simple linear electrical properties. They are simple, relatively large holes that don't pass proteins or nucleic acids, but do pass small ions and a lot of the small molecules involved in intermediary metabolism (2).

Turin and Warner (3) made an interesting discovery. If you acidified the cell cytoplasm, you uncoupled the cells; that is, you greatly decreased the conductance of the gap junctions. The uncoupling is due to cytoplasmic acidification because it is only produced by weak acids, such as CO_2 or acetate, and the undissociated form of the weak acid can cross the cell membrane. As Dr. Tsien was talking about this morning in terms of calcium chelators; a neutral molecule can cross the membrane and dissociate inside the cell, releasing hydrogen ions. Bathing in strong acids to which the cell membrane is not permeable does not cause these changes. So it is intracellular pH.

Naturally, we wanted to know what the relation between intracellular pH and junctional conductance was. By working with pairs of blastomeres, we could unequivocally measure junctional and nonjunctional conductance, and so show the relation directly between junctional conductance and pH.

Plotting junctional conductance as a function of pH, we obtained a simple relation (Figure 2). The triangles with an apex down represent initial conditions and cytoplasmic acidification. The triangles with apices pointing up represent recovery. There is a very close fit between conductance going down and coming back up again. There is very little hysteresis. From the absence of hysteresis, we tend to conclude that there is not a cytoplasmic intermediate involved, that it is a direct action of the hydrogen ions on the channels. If not, there must be very rapid equilibration between some cytoplasmic intermediate and the intracellular pH.

Another point here is the fit to the smooth curves, which are Hill plots for a Hill coefficient of four and five, which is what would happen if the hydrogen ions were acting on a single molecule with four or five sites in a highly cooperative fashion. Alternatively, there might be more sites with a smaller degree of cooperativity.

Other points to notice: The resting pH is very close to where cytoplasmic acidification causes a decrease in junctional conductance. The conductance is really quite sensitive. There is a dramatic drop with a change of a few tenths of a pH unit. The halfway point, the pK_H, is roughly 7.3, or 50 nanomolar.

Now, there was data in the literature previously, convincingly, from Rose and Lowenstein (4) -- that increase in cytoplasmic calcium would cause a decrease in junctional conductance. So the question arose whether the change that we were seeing with pH was actually due to change in cytoplasmic calcium. We did experiments with Joel Brown using aequorin and could see no increase in aequorin luminescence during acidification adequate to uncouple the cells (5). Luminescence would have been expected to occur at micromolar levels of calcium. Rink, Tsien, and Warner (6) did similar experiments using calcium-sensitive microelectrodes and saw no increase in intracellular calcium when cells were uncoupled by cytoplasmic acidification.

That left us with another question: Does calcium actually act on the junctions or is the effect of calcium one of pH? Putting calcium inside cells there might be an exchange in intracellular buffering mechanisms, as the cells scarfed up calcium or got rid of it that caused a release of hydrogen ions and cytoplasmic acidification. The work of Rose and Rick (7) suggested that calcium would uncouple cells without any change in cytoplasmic acidification. We also wanted to look at that, which we did, working with Jeff Stern, who brought a method of intracellular perfusion or dialysis that he had developed working with John Lissman.

What we observed was that the pH sensitivity of the conductance of the perfused membrane was essentially the same as that observed with intracellular pH microelectrodes. When we went to work with solutions now buffered to pH 7.8, but at different calcium concentrations, the membrane was extremely insensitive to calcium ions (8). It takes calcium concentrations of tenths of a millimole to affect the junctional membrane. So Roger is not the only one who is coming to a calcium meeting and telling you that calcium isn't very important -- not, at least, in the control of junctional conductance when calcium acts from the inside of the cell.

This is not to say that calcium doesn't ever act on the inside of the cell. This is a ridiculously high and unphysiological level of calcium, as has been emphasized by several speakers. The cell would explode or destroy itself with that kind of intracellular calcium. But there is a very important pathophysiological response when cells are coupled together by gap junctions. If one of the cells is killed, it uncouples from its neighbors. The conductance of the gap junctions goes way down.

One of the ways -- you can imagine how we found it -- of getting cells to uncouple is to make a little hole in one of them, letting in the external medium or letting out something that is inside. But a very good candidate for something to get in and cause the cells to uncouple, that is to decrease the junctional conductance, is the calcium in the extracellular solution. So calcium can be operating in a pathological way, where the surface membrane has broken down and intracellular calcium rises greatly. Such a mechanism can be important in the functioning of the organism in protecting it from that kind of a cellular injury.

The action of hydrogen ions is obviously a great deal more sensitive. They are about 10,000 times more effective in altering junctional conductance than calcium. So in terms of physiological changes that modulate the junctional conductance, hydrogen ions are a much better candidate for an intracellularly applied control than are calcium ions.

I probably should address the question of how this fits with the data of Rose and Lowenstein (4), who have, for a long time, pushed the role of calcium in controlling junctional conductance. It is hard to do quantitative work with aequorin, and I don't think they ever really tried to quantitate their aequorin results. The data that they have based their quantitative estimates on are from Olivera-Castro and Lowenstein (9), who did the experiment of making small holes in a cell while changing the extracellular calcium. The data that they give is that, between 40 and 80 micromolar junctional conductance begins to fall. They did not do anything in terms of buffering their calcium concentrations. It might be rude, but not unreasonable, to suggest that there may have been calcium above that level in their solutions, either from their "distilled" water or from whatever contamination source.

So there really isn't such a dramatic difference in the calcium sensitivities of junctions in our tissue and in their tissue, the Chironomus salivary gland. Their sensitivity is still very far away from what they also see for hydrogen ions, a sensitivity in the range of pH 7.

By now, the pH sensitivity of gap junctions has been demonstrated in a moderate number of tissues of both vertebrate and invertebrate origin, and both neural and non-neural. There are a few instances where people have been unsuccessful in obtaining a decrease in junctional conductance, or a change in coupling rather, with bathing in weak acids. Cases included junctions between lens fibers and between rods in the toad retina. These are situations where intracellular pH measurements and junctional conductance measurements are difficult to make and have not been adequately made. So there really isn't a titration curve in those cases yet.

It is not impossible -- and I would say it is pretty likely -- that there are differences in pH sensitivity in different tissues. This is a suggestion which we initially didn't like and have been resistant to it for a while. There are now some chemical treatments, like treatment with glutaraldehyde or EEDQ or retinoic acid, that greatly reduce or block pH sensitivity in the region where it is conveniently changed by bathing with weak acids (unpublished data with Spary, Calvalho, White). So it is reasonable that a relatively small change in the membrane macromolecule, which could be produced evolutionarily, has produced differences in pH sensitivity in different tissues. But certainly pH sensitivity is very widespread.

Before I forget -- and I have already forgotten -- let me, at this natural break, point out that Dave Spray is responsible for a great deal of this work, who is sitting here and whom most of you will have an opportunity to talk to. Certainly you can question him about any of it as well as you can question me, in the course of discussion and so forth. He certainly gets a grea deal of the credit for what I have been talking about.

Now I would like to go on to something completely different, another method of controlling communication at gap junctions.

From eggs of the amphibian Ambystoma Mexicanum, you can pull out pairs of cells and do the same kinds of experiments as described previously in this talk. After all the data on electrical linearity of gap junctions that had been gained over almost two decades -- 15 years, anyhow -- it was a bit of a surprise to find that these junctions behaved differently. They weren't linear at all. Well, for small currents, they were fine. However, with larger current pulses, the potentials of the cells changed in a way suggesting that the junctional conductance was changing during the pulses.

Well, this was a natural thing to investigate with voltage clamp, because there were time variant changes. But since we had two cells, it was necessary to have two voltage clamps. What we did -- and Andy Harris was a major collaborator in this work -- was to voltage clamp both cells, having them both at the resting potential, and then stepping one cell to some new value and holding it there. When the voltage step is made in this situation, current starts to flow through the junctional conductance, and current is applied by the voltage clamp on the second cell to keep its potential constant. So the current supplied by the second clamp is equal and opposite to that flowing across the junctional conductance, and we can measure the transjunctional current as this clamping current. There is no change in current across the nonjunctional membrane of the cell clamped at its resting potential, because its potential remains the same.

So we have a simple record, when we apply a step to one cell, of the current through the junctional membrane, and we can get the conductance as a function of time simply by dividing by voltage. It is constant of proportionality. One you can see by inspection, the time course of the conductance change.

Representative voltage clamp records are shown in Figure 3A. A small (Y_a, upper records) voltage in one cell (V_a, upper records) produces a small current through the junctions (I_j) -- that is, the current being applied in the cell whose voltage is V_a -- and the conductance is constant over time for that small voltage.

For a larger voltage (lower records), the current rises abruptly when you apply the step, which represents the resting conductance. Then I_j, proportional to conductance, drop exponentially down to a low level.

When one plots the steady-state conductances as a function of transjunctional voltage, one gets the relation shown in Figure 3B. Conductance falls steeply on either side of zero transjunctional voltage. The smooth curve, is derived from the Boltzmann distribution, which is what would obtain if the channels that make up the gap junction could exist in either an open or a closed position and the energy difference between open and closed states were a linear function of membrane potential. Near zero voltage most of the channels would be open; for large voltages on either side, most channels would be closed, and varying numbers would be open and closed in the transition regions. We can get a very good fit of the Boltzmann relation to the points that we actually measure.

So the hypothesis is that the channels do exist in either open or closed state and that the energy difference between them is linearly affected by potential. Relations of this kind are perfectly reasonable for membrane macromolecules, including excitable channels.

The decays of current over time are exponential (Figure 3A, lower records). This form is consistent with a first-order process of closing, where the probability of a channel's closing is constant over time, and it closes very rapidly with respect to the time course of decay. That is, it goes suddenly from an open condition to a closed condition. The time course, thus is also consistent with an open-and-shut kind of channel with a rapid transition between the two states. We model, then, the membrane channels as having a gate with some charges present on it, and these charges move around in response to a transmembrane field, leading to a redistribution with a change in dipole moment in the direction of the transjunctional field.

One would like to relate the voltage gate to the pH gate. Are they the same or are they different? Chemical tests suggest that they are different. You can affect one gate without affecting the other gate. For example, if you uncouple the cells by acidifying the cytoplasm, you do not affect the voltage sensitivity of the residual conductance. That remains the same. There doesn't seem to be any interaction between the pH action and the voltage action. Because of a variety of chemical treatments, which are now fairly numerous, we believe that there are two gates, really two kinds of gates, for there may be one of each of the two opposed membranes.

Here, then, are two mechanisms whereby gap junction channels can be open and shut -- more than two in terms of the number of agents that act, but probably through two distinct gating mechanisms. There are places where one thinks the gates do have some physiological significance in controlling intracellular communication through these channels. In terms of space and weightlessness and things like that, I don't think there is anything to be said about what the gap junctions are doing.

What we have entirely left out, and what we have really explored very little, is the whole question of the gap junction's formation and disappearance. We don't know where these membrane macromolecules come from. We know junctions can form in a few minutes. We know that in some instances cells make them go away. For example, cells transiently form gap junctions during embryonic development, and similar responses occur in certain adult tissues (10).

In summary there are the gating mechanisms, which are rapid, involving hundreds of milliseconds. Then there are the formation and dissolution of the junctions. The junctions can come and go away, and the cellular controls thereof are something that we still have no handle on. These controls may be more important in the kinds of long-term changes that one is concerned with in a symposium of this nature. One should be aware that they are there, but we yet don't know much about them.

Thank you.

DR. ROUX: Any questions?

DR. ROBINSON: Can coupled cells maintain a sufficient potential difference between them to close the gap?

DR. BENNETT: Yes. That is easily demonstrated experimentally by making a hole in one cell reducing its resting potential of the cell, and the leak in the cell will allow the cells to be stably coupled and uncoupled.

DR. WATTERSON: I have two questions, partly out of confusion. You said that calcium is not involved in junction communication. Yet you said, I thought, that there was an effect a few tenths millimolar C_a.

DR. BENNETT: Yes, right. I said that I don't think that calcium is a physiological control for junctional communication. The concentrations required are much too high for ordinary circumstances. If you do get calcium very high, as can happen when you make a hole in the cell, then the junction will shut down.

DR. WATTERSON: An analogous situation came up yesterday with enzymes. I think the point was made that locally around the macromolecule you can have quite distinct concentrations versus away from it. Is there any evidence as far as gap junction structure and what the calcium concentration is around that? Especially if it is involved in transferring across, is it not feasible that you could get up to millimolar concentrations?

DR. BENNETT: I don't think anyone thinks that there is ever a transient of that magnitude associated with physiological channels that let calcium in.

DR. WATTERSON: Secondly, could you summarize the data that demonstrates that the polypeptide everybody sees is the same thing as the EM structure, and that EM structure is the same thing that you are measuring by electrophysiological techniques?

DR. BENNETT: Item one: Why does the protein come from the gap junctions? Because there is just so much nitrogen in the gap junction fraction, and when you run it on a gel, there is essentially one band. There is nothing else there.

Why is a gap junction a site of electrical coupling and of dye coupling? The result is partially inductive. That is, if you don't have gap junctions, you don't have coupling. If you have gap junctions, you have coupling. You can show with dye that it will go from one cell cytoplasm to another cell cytoplasm, but that it does not cross the extracellular space -- or, more accurately, it doesn't get into the cell when applied from the outside. Thus there is a private pathway between the cells that is not open to extracellular space. The only place the cells get close enough to do that is at the gap junction.

DR. WATTERSON: Has anybody been able to reconstitute a gap junction?

DR. BENNETT: No, not yet. Hertzberg thinks that he has got intra-membrane particles into liposomes from a protein fraction. I don't think anyone has made a reconstituted gap junction with two membranes.

DR. SPRAY: No, but we have patched onto isolated junctions membranes.

DR. BENNETT: What Dave is talking about is that we have recorded single channel currents by patch clamping membrane preparations from lens. These membranes have a lot of protein, but there is some question as to whether it is gap junction protein or another protein. The major lens protein forms morphological structures (at EM level) that are distinct from these in the usual gap junctions. This protein or another protein in the membranes may make channels, but gap, and we don't really know what we are recording from.

DR. WATTERSON: I would inject here that I have analyzed Hertzberg's liver gap junction protein preparations, and chemically they vary from prep to prep, and functionally, as far as calmodulin binding _in vitro_, they vary from prep to prep.

DR. BENNETT: I don't want to get into protein isolation. You fight it out and tell me.

DR. WATTERSON: I want to know, do you really believe that that major polypeptide is that EM structure?

DR. BENNETT: What I believe is irrelevant. I would like to believe it, but I am not sure I am as critical about it as I ought to be.

DR. TSIEN: Has anyone made antibody against it or anything?

DR. BENNETT: It is not very antigenic. There are some antibodies around. The liver gap junction protein, an awful lot of it has gone into rabbits, very little has come out.

DR. SLOCUM: I just wanted to know whether you thought that calcium might be involved in maintaining, or maybe establishing, these associations between the membranes?

DR. BENNETT: That is perfectly possible. An absolutely standard way of dissociation cells is in calcium-free solution. There is some evidence, unpublished I believe -I haven't seen it yet, and I only know it basically from Dave's talking to John Heuser -- that in calcium-free solution gap junctions split down the middle. Calcium may be required for maintenance of junctional structure. On the other hand, there are other preparations treating intestinal epithelium or gastric mucosa with calcium chelators. The last thing to go is gap junctions. Some cells fall apart in calcium-free, others seem perfectly stable. So I don't think all the data are in on that. But a role for maintenance of the junctional structure by calcium in the extracellular space is still possible.

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BENNETT FIGURES

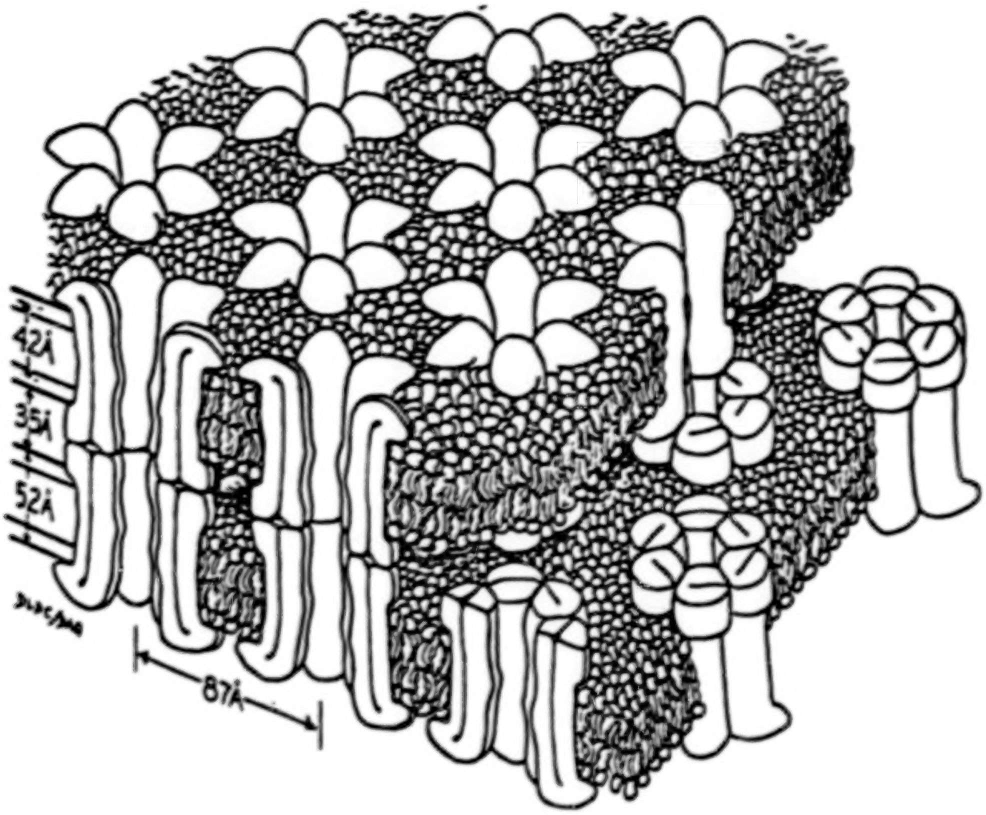

FIGURE 1. Diagram of the structure of a gap junction. The c. 6 nm in the diameter channel units (connexons) are shown floating in the two opposed lipid bilayers. Center to center spacing is 8.7 nm. Each hemichannel is a hexamer of the constituent protein subunits. Several channels are shown in longitudinal section and in cross section in the middle of the gap. From Makowski, et al., 1977 (11).

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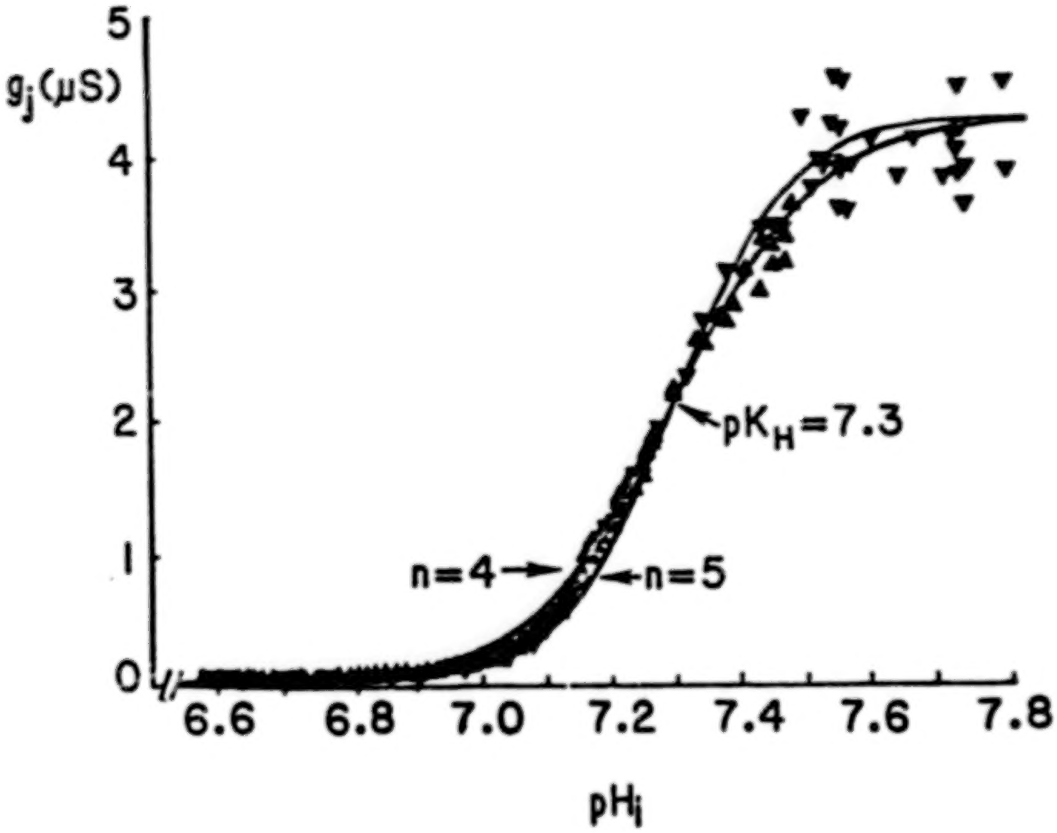

FIGURE 2. Control of junctional conductance by transductional voltage. A. Decreased junctional conductance during transjunctional voltage steps. Each of an isolated pair of blastomeres was voltage clamped with two microelectrodes. The potential in one cell (V_b) was stepped from the holding potential and junctional current (I_j) was measured as the current supplied to the second cell in order to keep its potential (V_a) constant. For this system, junctional conductance g_j is given by I_j/V_b. For small transjunctional voltage steps, g_j remained fairly constant. For larger transjunctional voltage steps of either polarity, g_j fell exponentially to a steady state level. From Harris, et al., 1979 (12).
B. Relation between steady state junctional conductance g and transjunctional voltage V. The steady state conductances were determined for different transjunctional voltages from data like that shown in A. The relation between g_x and voltage is steep, and is well modeled by equations describing a Boltzmann distribution of conductance elements between open and closed states where the energies of the two states are simple expontential functions of voltage(solid line)(13).

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Figure 3a

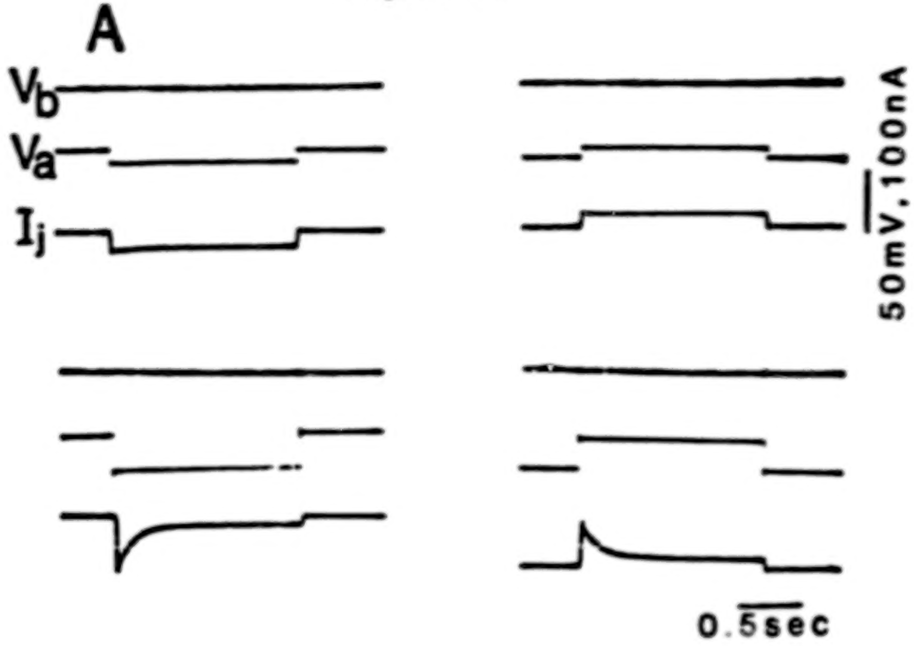
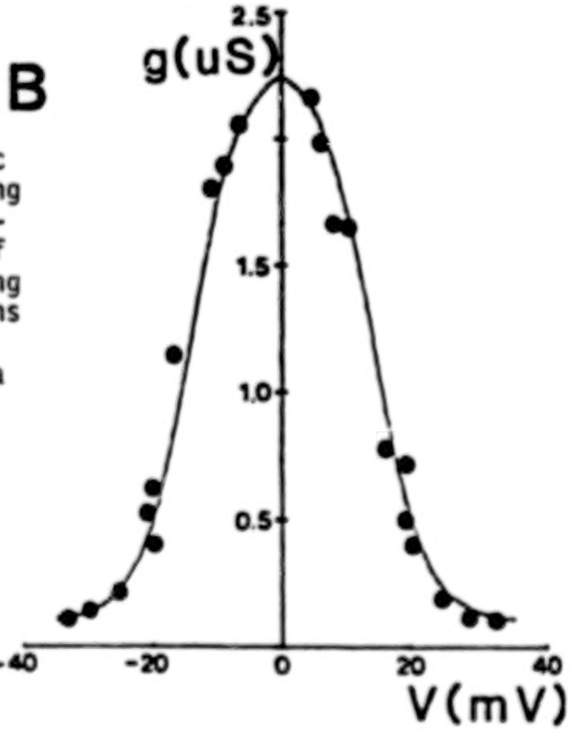


FIGURE 3. Sensitivity of junctional conductance to intracellular H ions. Conductance data are plotted as a function of pH. Triangles with apices up are values during recovery. Triangles with apices down represent initial values and values during cytoplasmic acidification. The points fall along Hill curves with pK_H = 7.3 and n between 4 and 5. There is evidence of hysteresis between falling and rising pH_i. The data consistent with H ions acting directly on a channel macromolecule. From Spray, et al., 1981a (14).

Figure 3b



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DR. ROUX: Thank you very much.

We are now kind of entering into the meat and potatoes of this workshop. I want to give you the briefest possible overview of what we are going to be doing in these discussion group sessions.

We have had, over the last day-and-a-half, the opportunity to hear people speak about the role of calcium, or the non-role of calcium, in various physiological responses. We have also had a chance to hear gravitational physiologists speak about the various unsolved problems in understanding the effects of gravity on plants and animals.

What we want to do in these discussion group sessions is to pool our common experience and think about some of the problems in gravitational physiology, and ask whether any of the things we know about calcium metabolism and function might pertain to some of the problems that we are trying to solve in gravitational physiology.

So that is the overall goal of these discussions. The actual format will be that we will meet in groups of six, in small areas which I will define in just a minute, with discussion group leaders, who have prepared trial balloon questions for each group. These questions were prepared with the composition of the group in mind, with the expectation that they would elicit discussion. It may turn out that they will be lead balloons, and those discussions won't really turn out. But then any other discussion, of course, will be okay, perfectly fine for reaching the goals of the discussions.

DR. ROUX: Now we will have the reports of the discussion group leaders. We will go in the order in which they are listed here on the sheet. That means Dan Bikle, would you start us off?

DR. BIKLE: My discussion group was comprised of Drs. Rasmussen, Holton, Spray, Robinson, and Malacinski. The question that I posed to the group was, I wanted them to consider the role of gravity on the problem of bone response, to address the problem that NASA faces, when it sends man into space, of the rapid and progressive bone loss that occurs. I asked them to think of the types of signals that might be mediated by gravity, or loss thereof, on bone response, bone formation, bone activation.

Well, that is where we started. We considered briefly the fact that bone itself could act as a transducer of electrical effects, which might somehow regulate cell function. The question as to how that might regulate cell function was then discussed, and no answers were forthcoming. I think the bottom line that this group reached in terms of this problem is that we need considerably more research into defining a basic model which would permit us to study a variety of possible mediators of effects on bone action. Quite frankly, the models that are currently available to us were not deemed to be satisfactory at this stage for much progress in terms of the question of gravity on bone loss in space.

Basically, we are way back, in a very primitive state of knowledge, and we need to bolster that before we can move much further on this question.

That is all I am going to say.

DR. ROUX: No suggestions as to where to start?

DR. BIKLE: Yes. The first halting steps that were suggested are, we probably need to determine, number one, the responses of different types of adult bones, which we could manipulate in vitro, in culture. So tissue culture work, of all the ideas that were suggested, was probably the number one place at which we would start, and develop good models which would permit manipulation, say, of electrical fields, to see what would happen to bone.

There are a number of problems with the currently available tissue culture results, not least of which is that it is very difficult to have adequate perfusion of an intact piece of bone. We know also that blood-borne elements are very important, for example, monocytes entering bone and stimulating or becoming the osteoblasts. The types of in vitro models that we have are really not a very good simulation of the situation as it exists in vivo. Bone has a lot of technical problems in terms of its study in vitro that really must be addressed before we can go much further.

Any of my group want to amplify or correct or amend anything that I want to say? Is that a true reflection of where we were?

DR. ROUX: Maybe it is premature, but any opinions expressed on whether calcium might or might not be involved in the whole process?

DR. BIKLE: No opinion was ventured on that. I think we were staggered by the enormity of the ignorance that we had, to even try to attempt to identify any particular mediator.

DR. ROUX: The next group leader is Bob Bandurski.

DR. BANDURSKI: Our group was a moderately diverse one, composed of Lisette Lagace, Bennett, Chafouleas, Ross, Zielinski, and myself.

We began with the generalized question, what can only be done in a low-gravity environment? And can we design experiments that could, and really should, be done under these conditions?

We spoke of one thing, and that is nausea, and wondered if we know enough about nausea, whether or not space nausea is sufficiently different from normal motion-induced sickness so that they are things that could only be done in that way, and particularly whether or not we could devise animal systems. I think we agreed that cats, for example, with a bissected brain, or monkeys, where nausea can be induced, might be very good model systems. Again, if we begin to think of prolonged space travel and the desirability of not having somebody up there who is sick most of the time, this might be a very, very important thing. They told me that human beings are very variable, and very few get to do it a second time. So it is too bad if you come back and say, "Darn it, I missed it all." So we would like to try to avoid that and learn more about motion sickness, using animal models.

Then we talked a little bit about trying to devise either a cell culture -- and I see that has already been mentioned -- tissue culture things, or very short-lived animals, for example, nematodes, where we have an extensive DNA library and a great deal of mapping has been done. Can we then devise systems that can be used not only to look for possible radiation-induced damage, or maybe a Bruce Ames type test, using Salmonella -- but in every case, we were looking for ways of simulating mammalian and higher plant changes using either microbial or tissue culture systems. Muriel had the suggestion that one could also use rapid healing of a broken bone, for example, as a study of decalcification. But we also would like to look maybe even at microbial things with a calcareous or siliceous structure and see if they respond to low g.

Our philosophy in this case is that, even if they don't, it would be a lot cheaper to do this experiment with microorganisms and tissue cultures, rather than do it as one of our sister agencies did, with soldiers on Frenchman's Flat. So we would like to do that.

Then there are other questions. For example, do aquatic animals decalcify? Could we use Dr. Tsien's chromophoric monitoring of calcium to look specifically at decalcification in a tissue culture or in a cell line in space? Could one even begin to simulate the kinds of instrumentation that would be required?

Then we spoke of the general question of, do we really know enough about a zero environmental input system? You know, can we really say that an organism being grown essentially at zero g and essentially without light, and at constant temperature and constant humidity — do we know how creatures would respond to that type of environment? I think Dr. Bennett said probably that is a high-risk experiment. We think nothing will happen, but, on the other hand, he concurs that it probably should be done.

There were other questions. We could find out whether or not decalcification was at all related to the postmenopausal decalcification. Can we use some of these changes as models?

Then there were other things. Particularly Dr. Zielinksi and Lisette were interested in monitoring for specific DNA changes. You know, we shouldn't just confine our attention to chromosomal changes. We should begin to look for things that will perhaps give us a somatic change at the DNA level. Again, we thought of the nematode system, because of its library, or the microbial system, or even the Bruce Ames type of monitoring with Salmonella. Perhaps we should view space as being one more mutagen, both in terms of zero g and in terms of radiation input, and begin to look for compact and cheap and easily monitored systems, such as the Ames test has been for microbial mutagens.

Then we talked a little bit about the unique involvement of calcium. Again, we thought of DNA replication, and again of NAD kinase, and the possibility here of monitoring transitions, say, between DPN or TPN system. I think one could design microbial models that would make for this kind of thing. Again, using the quin 2 type chromophore, I think perhaps these things could be simultaneously correlated with ambient calcium concentration in the cellular or microbial culture.

DR. MALACINSKI: What is the best guess concerning the answer to the question of whether aquatic animals would decalcify? What is the guess?

DR. BENNETT: I guess that they wouldn't.

DR. KEEFE: You mean in null gravity? Fundulus has been flown for 55 days duration, and no significant decalcification could be detected.

DR. MALACINSKI: Because the experiment I want to suggest is, for example, the salamander that undergoes metamorphosis. If this is an aquatic form, if your prediction is correct, then it won't undergo any sort of decalcification. But it can be induced to undergo metamorphosis rather simply. But, now, the terrestrial form presumably would. Once it undergoes metamorphosis, it now starts to decalcify. I am suggesting a comparison between the bone structure in the form that is predisposed to decalcify as opposed to the one which is not.

DR. BANDURSKI: By the way, there is a remarkable analogy between the animals and plants. If you weight-load a tree, it builds up stress wood. We don't know how the tree feels that weight, in the same sense that we don't really know how an animal feels the weight or lack of weight on its skeletal system.

DR. CLELAND: Have land snails ever been flown in space? It would seem to me that there is a calcified system where you could compare land and water snails, and look for decalcification there. Or is the shell of a snail never decalcified?

DR. BANDURSKI: There are things that shed scales, like crabs.

DR. KEEFE: It seems to me Helix has been flown, but I am not sure. I don't remember any of the details.

DR. BANDURSKI: Well, we didn't pretend to have the expertise to actually say whether the experiment was doable or not. But I guess the take-home lesson, if we do have one, is that we just have to look for microbial and cultural systems, cell tissue culture, and quick-responding systems, like a broken bone that can be broken in space and partially healed in space, and to look for things that will give us more data per buck, in shorter time.

DR. ROUX: I have a question that might be addressed to Ray and Jim, and maybe Lisette. What is known about calcium regulation of gene activity? I should know, but I don't know. What is the latest information on that? If there were major alterations in, say, calcium metabolism or calcium-sensing systems, would that have an effect on gene regulation?

DR. CHAFOULEAS: In myogenesis in culture, where you can put cultures on a myoblast, you can totally inhibit that in the expression of the actin gene by chelating out calcium in the culture. So that is a very good well-defined system for the role of calcium in gene regulation.

DR. TSIEN: Is it specific to actin?

DR. CHAFOULEAS: For myogenesis it is, yes.

DR. ROUX: What is the kind of experiment?

DR. CHAFOULEAS: You take chicken myoblast cells, put them in culture, and under proper conditions, the cells will then fuse and form myotubes. There is a rapid increase in actin biosynthesis, and it is coincident with myogenesis. This is calcium-dependent. You can deplete the medium of calcium, and you don't get actin genetic expression.

DR. CLELAND: Will the other developmental events go on?

DR. CHAFOULEAS: No. Everything stops. But actin is a major marker.

DR. TSIEN: There is a report from Columbia using GH3 pituitary cells in tissue culture, where calcium removal or a reduction of extracellular calcium stops the expression of the synthesis of one hormone, but leaves all the other hormones okay, and the cells are happy. So even that differential switch can be done, as opposed to a rather larger scale growth arrest. They have various blocks and so on. I forget which transcription or translation stage it is.

DR. ROUX: Is that work in press or has it already been published?

DR. TSIEN: It is published in the Journal of Biological Chemistry, not so long ago.

DR. BANDURSKI: They also found CHO cells, too, where you could obtain a synchronous culture and it could be very readily done, on even a short-duration flight. Three or four days would be enough to look for changes in synchrony or changes in DNA, et cetera.

DR. TSIEN: I have lost track. What does that have to do with gravity?

DR. BANDURSKI: Well, if it is in zero g, and if there is a direct effect upon calcium-mediated processes in the cells --

DR. TSIEN: Shouldn't we find out if the extracellular environment changes calcium in response to gravity? Shouldn't we find that first? I was amazed to find that no one had ever checked the blood of astronauts.

DR. HOLTON: Not for ionized calcium. They have done total calcium. If you look at the latest data I have seen, there is a slight increase, but it never gets above what one would consider normal levels. It is supposedly significantly higher, but it is not that much higher.

DR. BANDURSKI: In the urine or in the serum?

DR. HOLTON: In the serum.

DR. ROSS: I think you forgot one thing that we discussed in tissue culture, and that was your idea about some particular cell where you could study them both in a spherical stage and when they were down on the surface, and see whether or not gravity had an effect on the arrangement of actin and microtubules.

DR. BANDURSKI: That was the other thing, yes. We wanted to focus a lot of attention on microtubules, because if you have a calcium role in tubulin, then I think if you can monitor cellular calcium and look for changes in tubulin, you would be far ahead, as in the CHO system.

DR. WATTERSON: One suggestion I would throw out related to both these points is the multiple actin genes of Dictyostelium, where what is expressed depends on the arrangement in the organism's developmental stage and orientation. So you have a simple system, inexpensive to grow rapidly, to look at things, such as multiple gene expression.

DR. HALSTEAD: There is just one point. From the few experiments that have been flown on microorganisms, on bacteria, they have not seen changes. So it is at least hypothesized that the size of the organism is too small to show --

DR. BANDURSKI: The question is whether a microorganism would show gravity effects. But others have indicated that we don't really know that yet.

DR. HALSTEAD: I agree. I am just throwing that out.

DR. ROUX: Bob, as a background to that, do some people believe on theoretical grounds small organisms could not respond to zero g?

DR. BANDURSKI: Originally they were thinking that simply the dimensions of the organism would be such that the transient across the dimension of the organism would be so small as to preclude any change. You had to have a cell of a certain size before you could experience anything.

DR. KRIKORIAN: But T4 phage was tested, remember? And it was affected, the reassembly of the virus.

DR. HALSTEAD: Everything contradicts everything else.

DR. HEPLER: On that size issue, I would expect it is somewhat similar to the situation where, when you have a very small organism like that, the viscosity of the fluid would greatly override the gravitational effect.

DR. BANDURSKI: Yes, and, nonetheless, you have heavy organelles.

I wanted to apologize. My group gave so many ideas so rapidly that I couldn't possibly write them down, so I am sure I have missed many things.

DR. HOLTON: They have also flown lung cells in culture, which really showed no changes. They looked at a lot of things. The only difference was that the cells in space utilized less glucose, which may have been an artifact. They don't know. But other than that, anything else they looked at in lung cells was normal -- and so they wondered whether gravity effects would be perceived at a cellular level or whether it required organs or more integrated cellular structures for gravity effects. That is the only cell culture work that has been flown.

DR. BANDURSKI: These are long-lived cells. They would have a long division time, too.

DR. CLELAND: I was really impressed. That seemed like a very excellent, thoroughly done study with animal tissue culture cells, showing absolutely no real effects.

DR. KRIKORIAN: Except -- I have it in front of me here -- the leakage of glucose into the medium in the flight units was in fact greater than in the control.

DR. HOLTON: But everything else they looked at, even the enzymes and things which should have been influenced by glucose, were not.

DR. KRIKORIAN: Well, except, again, if you look at things like individual length of cell cycles and so forth, the data is not all that clean. I think there was a tendency to interpret it the way he wanted to at the time. Happily, the data is here, for anybody who wants to go back and check it. It was published in In Vitro, Volume 14, 1978.

DR. BANDURSKI: I should say also that the generation time for lung cell culture would be relatively long compared to the types of things we are talking about, plus the fact that with Salmonella you could monitor DNA changes as well, with a good library.

DR. ROSS: I really should speak up for what you suggested. The nice thing about what he suggested, as I understand it, was that you had a cell that could be spherical and one that could go down on a grid and flatten out, and you can look at things like microtubules and actin configurations, and you can look at a gene, and all the rest of the stuff. To me, that was the nice thing about what he suggested, the same kind of cell grown under two totally different conditions.

DR. ROUX: Thank you, Bob.

The next group leader is Bob Cleland.

DR. CLELAND: My group contains Marty Watterson, Roger Tsien, Stan Roux, Peter Hepler, and Meryl Christiansen. Since we are interested here in this workshop on calcium and its possible involvement in various physiological and biochemical processes, which might or might not be affected by gravity, and since most of us in this group have been primarily interested in plants, an area in which there is a good deal less known about calcium and calcium control and involvement than in animal systems, we asked the question: How would you go about most effectively simply measuring some of the parameters that one simply has to measure if you are going to ever really understand what is going on, particularly in plants?

I simply point out, for example, that, to the best of my knowledge, in the higher plants there are no measurements of intracellular calcium in the cytoplasm. There are no really good measurements of it in the extracellular cell wall space. Obviously we need to know what these values are, whether we are talking about 100 nanomolar or one millimolar calcium in the cytoplasm. We can make guesses, but we need to find out.

So we talked about various methods that might be possible, and discussed the pros and cons of them. We talked about the use of the quin 2 type dyes. It was pointed out that, while these would simply have to be checked out to find out whether they are a possibility, we may have problems because of the multicellular nature of plants in getting enough material in and getting the optical path such that you could actually make the necessary measurements. But that is one of those things that will obviously have to be looked at in the near future. Since these dyes are now apparently available, according to Roger -- Amersham now is selling both tritiated and nonradioactive quin 2 --

DR. TSIEN: I don't know that. In July someone told me that they thought that Amersham was about to start.

DR. CLELAND: Oh, okay, it is an unsubstantiated rumor.

We talked about other possibilities. One that people have used is the EDAX electron microprobe method. It is probably not sensitive enough to really be particularly useful to answer the kinds of questions that we are interested in here. Another much more promising technique is to make use of lasers to volatilize a very small area of frozen thin section, which then could be analyzed for calcium in mass spec. The problem here is that, as far as we know, there is only one machine of this type in the world, in Europe somewhere.

DR. WATTERSON: Somebody said Heidelberg.

DR. HEPLER: That is the proton microscope.

DR. CLELAND: How about the laser one? We are not sure where at this point. But it may be very promising because it can use a very small area, and it has a good deal more sensitivity than the EDAX.

We talked about the possibility of using micro-electrodes in plants to measure extracellular, the cell wall calcium concentrations. We concluded that this was probably going to be difficult at best, to get the electrodes into the right place to make the kinds of measurements that are wanted.

Another approach to doing the same thing would be simply to centrifuge out the extracellular wall solution from a tissue and then analyze it directly. This might be a more effective way of measuring the free soluble calcium level.

We talked about the antimony precipitate method and concluded that this is probably not a very quantitative measurement, and we aren't sure whether it measures free or free and bound calcium, for example. That is one of those things that probably needs to be determined.

We talked a little bit about the measurement of calmodulin, if we wanted to know whether a calmodulin might be involved in a particular response in plants. We concluded, I think, that the radioimmunoassay is probably one of the best ways to assay for it, but that there are definitely problems involved. The nonstandardization of the antigens, the necessity to test these things out very thoroughly and know what you are doing, means that it is not something that probably will be used by a large number of people. The problem is confounded by the fact that there definitely are other calcium-binding proteins in plants, and one cannot just simply look for calcium-binding proteins and assume that you are looking for calmodulin.

The use of the phenothiazines as inhibitors to test may be very useful, but again one has to use extreme caution to make sure that the effects that one sees are really due to effects on the calcium/calmodulin type system, and are not some sort of side effects.

This is about as far as we got. Does anybody on the panel want to add to that?

DR. RASMUSSEN: How much of that extracellular or extra-wall fluid can you get?

DR. CLELAND: You mean in terms of percentage?

DR. RASMUSSEN: In volume.

DR. CLELAND: If you take a number of sections, you can get out -- well, we have isolated, say, a ml of it. That is not very much, but it is certainly enough to do calcium assays.

DR. RASMUSSEN: No. I mean, if all the renal physiologists are using electron probe analysis to measure calcium and other ions in 25 microliters of glomerular filtrate, if you could get out 25 microliters of pure fluid by some device, they could measure it. Those things are all over the United States.

DR. CLELAND: There is no problem in doing that. The real question with that technique is whether you in fact are getting out something which is representative of the calcium concentration in the cell walls, in a tissue in general, and whether it is representative of the calcium in the region of the wall that you are most interested in, which is near the plasma membrane. We don't have any sort of independent way of testing for that.

DR. ROSS: I would like to ask a related question. I didn't think of it before, but does the quin probe measure total calcium, which is what you start out talking about, or does it measure the ionized or the free calcium?

DR. CLELAND: Ionized calcium, is my understanding.

DR. ROSS: It doesn't take something out that is bound.

DR. BANDURSKI: With regard to the quin probe, too, even that measures, really, not only free, but everything that has a dissociation constant larger than does your EGTA. So, again, in some ways, we still have not answered the question of measuring the calcium concentration in the immediate environment of either the membrane or the enzyme, say, in the wall.

DR. TSIEN: I am afraid you have a misunderstanding there. It all depends on the relative ratios of the buffer you use and the mmount of quin 2. Quin 2 can bind one molecule of calcium per molecule. So if you have one millimolar of buffer and one micromolar of quin 2 in, you cannot eat all the calcium that has been sitting on the buffer, even if that buffer is trying to maintain it at ten micromolar. All it will do is the quin 2 will say it is practically saturated with calcium. If you have very good measurements, you might be able to tell it is 99 percent saturated with calcium and tell it was ten micromolar. But you cannot strip all the calcium out of the one millimolar of buffer and pull it down to 115 nanomolar.

DR. EVANS: I don't think that is the question. I think he is saying that weakly bound calcium that would dissociate from, say, a protein or something had a pKa that would --

DR. TSIEN: But I just gave you an example. You have a very large reservoir of it, even if it has low affinity, and all it will do is saturate the quin 2, and the quin 2 will then stop taking up more calcium.

DR. EVANS: But surely you are not suggesting that calcium that is weakly bound and calcium that is bound by your chelator is not going to be in exchange. I mean, some of your chelator probe is going to give up calcium.

DR. RASMUSSEN: You are missing a very important point that Roger made, with his dye. I don't know about plant cells, but animal cells have this fantastic ability to buffer calcium. So when he puts quin 2 in there, transiently the ionized calcium is going to fall, and any buffers that are buffering calcium, there will be some dissociation. But the cell is going to readjust and retain calcium to make up for whatever calcium is bound to quin 2, so you are now going to be back to exactly where you were before you started, in terms of your buffer.

DR. TSIEN: Think in terms of pH. If we had had a molar concentration of strong pH buffer and I had put a few drops of phenolphthalein in, or some other indicator, and maybe it changes color at pH 7, do you think that, just because it changes color at pH 7, a few drops will necessarily rob everything else and force it to pH 7? You have to consider the relative ratio.

DR. ROUX: Bob, do you have a comment?

DR. KRETSINGER: Yes, I was just telling Howard that the example that was given about quin 2 is a question I have on my undergraduate biochemistry course, to point out to them that a sensitive indicator is not going to change the concentration of protons or calcium in a solution. And I promise them they can't pass the course if they don't get it right.

DR. ROUX: Okay, the next report is from Mike Evans.

DR. EVANS: It turns out that our group discussed almost exactly the same thing that Cleland's group did, so I can make it very short.

Our group consisted of Ronald dela Fuente, Chuck Caldwell, myself, Peter Kaufman, and Bob Slocum. We are primarily planty in orientation. We decided to consider the following two questions; First of all, in gravity tropism how is calcium redistribution accomplished? We also considered the question of whether or not, in fact, there is calcium redistribution during gravity tropism, as you see. What might be the relationship between the gravity sensor and the mechanism of calcium redistribution?

As a second question, we considered, how might asymmetric calcium distribution in plant organs be related to the asymmetry of growth that is seen in the response of plants to gravity?

With the first question concerning how calcium redistribution is accomplished during gravity tropism, one point that was brought up is that we have to be ready to admit the possibility that calcium redistribution in gravity-stimulated plant organs may not be a universal phenomenon. Peter Kaufman pointed out, for exmmple, that he does not find evidence for calcium redistribution in the grass Pulvini, and there are many systems that haven't been studied.

If we assume for the moment that there is general calcium redistribution in gravity-stimulated plant organs, we wanted to consider what might be some of the mechanisms behind it. Is it some primary response to gravity, or is it secondary?

One phenomenon we spent some time discussing is the observation that in gravity-stimulated plant organs there is, in all those systems that have been looked at so far -- and they are limited -- unilateral acid efflux from the gravity-stimulated organ. That is, in the case of shoots, there is acid efflux from the lower side, the rapidly growing side. In the case of roots, there is acid efflux from the upper side. We worried about the possibility that calcium redistribution might be tied somehow to asymmetric acid efflux, since we know that acid is very good at displacing calcium from cell walls. It is entirely possible that the thing that triggers redistribution of calcium is an asymmetry in acid efflux from the organs. It may be, for example, that acid secretion from the lower part of a gravity-stimulated shoot chases calcium out of the bottom of the shoot. That doesn't answer the question of how it moves specifically to the top. But we need to consider that possibility, and also to recognize that, if there is, in fact, acid gradient-induced redistribution of calcium, that doesn't mean that the calcium redistribution is not important to gravity tropism, but just that it may, in fact, be arising by gravity-induced asymmetry in acid movement across shoots.

As did Bob Cleland's group, we considered the importance of being able to measure gradients both in acidity across gravity-responding organisms and in calcium concentration, both in the apoplasts and synplasts. We considered the possibility of using hydrogen microelectrodes, calcium-specific microelectrodes, electron probe analysis, and so forth, and discussed some of the problems that would arise from those methods. Bob has already talked about some of those.

A point that was brought up that I think might be a particularly important one is that, as far as I know, in all those cases where calcium redistribution has been measured in gravity-responding systems, and asymmetric acid efflux has been measured in gravity-responding systems, the asymmetry is strictly dependent upon a source of the endogenous growth hormone auxin, and apparently depends upon redistribution of the hormone first. That is, it may be that both of these phenomena, asymmetry in acid efflux and asymmetry in calcium redistribution, are secondary to an initial redistribution of plant hormone.

The reason we focused on that is because it has been established that there are some very specific inhibitors of auxin redistribution in gravity-stimulated plants. Naphthyl-phthalamic acid and triiodobenzoic acid are a couple of examples. If you treat plants with these, you do not get asymmetry in hydrogen ion efflux, you do not get calcium redistribution, and you do not get gravity tropism. Of these three, the thing that seems to be most primarily related to the stimulus is the redistribution of hormone. I don't think we should lose sight of that.

We considered that, if calcium redistribution is dependent upon the acid efflux asymmetry, then it ought to be possible to use some of these so-called specific inhibitors of auxin-induced hydrogen ion pumping -- ATPase, orthovanadate, for example -- to prevent calcium redistribution, if the calcium redistribution depends upon asymmetric hydrogen ion efflux.

Then we considered one other phenomenon that has appeared in the literature. One of the members of our discussion group was Roland dela Fuente, who has some interesting data that hasn't been presented to this group. Essentially the observation is that in plant stems auxin movement in one direction, polar auxin movement, seems to be coupled somehow to calcium movement in the opposite --

DR. ROUX: For those of you who are not aware, auxin is a growth-promoting hormone in plants.

DR. EVANS: Thanks, Stan. Yes, this seems to be one of the major growth-promoting hormones. What Roland has noted is that there seems to be a relationship. If auxin goes this way, calcium goes in the other direction. We considered briefly the possibility that, since in gravity tropism-stimulated organs there seems to be a preferential movement of the hormone toward the bottom, and if Roland's data applies to transverse movement of the hormone, the movement of calcium to the top, as is seen in shoots, might be tied somehow to whatever the mechanism is for moving auxin unilaterally toward the bottom of a gravity-stimulated organ.

That is about as much as we said about the first question. The second question, as I mentioned, is the question of, if asymmetric calcium redistribution is important, how might accumulation of calcium, say, at the top of a stem, be related to reduced growth on the top of the stem. We considered two general categories of possibilities, one primarily physical -- that there may be some direct effect of calcium on wall loosening, direct effect of calcium on the growth process, either by the old -- which we don't believe -- classical ideas of divalent cation cross-linkages -- there is just too much evidence against that -- or we considered the possibility of some calcium effect on the affinity of cell wall hemicelluloses for celluloses, for which there is some evidence that this affinity between hemicellulose and cellulose might be related to wall extensibility. We considered the question of whether or not calcium might influence that.

In general, our group seemed to feel that these sorts of physical explanations were less likely, in part because of Bob Cleland's results that show that calcium doesn't do much in isolated cell walls. If you pull on them and try to increase their extensibility by applying acid or low pH, which is very well known to increase extensibility in plant cells, in a system like that, an isolated cell wall, calcium doesn't seem to do much.

As far as biochemical mechanisms, we came up with perhaps those that you would predict we might have come up with. Let me just mention a few of those.

One obvious possibility is the possibility that calcium somehow directly influences the activity of one or more potential cell wall-loosening enzymes. As far as we know, people have spent a lot of time looking at the pH dependence of cell wall-loosening enzymes in plants, and virtually no effort has been spent looking at the influence of calcium on the activities of these potential wall-loosening enzymes. That is at least one possibility for calcium gradients influencing growth gradients in plants.

Other possibilities we considered were calcium effects on the binding of lectin in plant cells. There are some people who feel that lectin cross-linkages in plants are related to wall extensibility. There is the possibility that calcium might influence the activity of what you might call cell wall-rigidifying enzymes. Peter mentioned the possibility that phenylalanine ammonia lyase, an enzyme which is involved in the biosynthesis of lignin, a wall-rigidifying component -- at least it is worth looking at the possibility that calcium enhances the formation of this wall-rigidifying stuff, lignin.

Another possibility related to wall rigidification, which I don't think our group really mentioned, but might be worth tossing out, is that there is recent evidence that, in parts of plants that are ceasing their growth, there is an increased incorporation of what is often referred to as hydroxyproline-rich protein, a wall component that seems to be related to wall stiffening. There is some evidence for an asymmetry in hydroxyproline-rich protein accumulation in gravity-stimulated organs, the slowly growing side containing more of the wall-stiffening stuff than the rapidly growing side. So we ought to consider also, I guess, the possibility that calcium might have some influence on formation of this wall-rigidifying stuff.

I can kind of summarize that part by saying, as far as potential biochemical effects, we are saying primarily that calcium might be able to -- this is obvious, I guess -- either enhance the wall-loosening aspect by stimulating wall-loosening enzymes or enhance what you might refer to as wall-rigidifying activity.

Those are the major things we considered. We did throw out one question that no one had an answer for. It is sort of a philosophical question. I would be interested in hearing the answer to it. Throughout the conference, we have heard a lot of data that calmodulin is good at turning on the activity of various enzymes in the presence of the proper levels of calcium, and we are wondering if there are any instances known in which calcium-calmodulin complexes shut off enzyme activity. Should we always be thinking in terms of an enzyme that is sitting there inactive waiting to be turned on? Or might we also consider the possibility that there is an enzyme sitting there active and it is going to be turned off when you allow it to bind with calcium-activated calmodulin?

Unless my group wants to add something, that is a summary of our discussion.

DR. WATTERSON: I can't quote any good data off the top of my head about inhibition, but I think it is a legitimate question, the whole concept of effector protein regulation. It is very important in enzyme assays to show that substrate goes to product. In most calmodulin-stimulated enzyme assays, people do not do that. I agree with you, we have to have a publication out emphasizing that. So I think that that is something that should be looked at.

As far as having one of the prototypes of effector protein regulation in biosynthesis, the lactalbumin glycosyltransferase system, there, if you weren't looking at substrate utilization and product formation, you would think that lactalbumin inhibited glycosyltransferase, and if you looked at another reaction, you would say it stimulated it. So that is a case in point.

It is not calcium, even though lactalbumin does bind calcium. There is no implication that calcium regulates there. But the protein type of regulation, effector regulation, there, you do have an apparent inhibition of one reaction and stimulation of another. So it is a good concept.

DR. KAUFMAN: Inhibition would be very nice for the wall-loosening enzymes.

DR. EVANS: That is right, if they are sitting there inactive and you can turn them on.

DR. KRETSINGER: I don't have a very clear model in my mind of the structure or dimensions of a cell wall, and particularly the enzymes within it. They are obviously extracellular. Are they covalently linked? Are they enmeshed? Are they in a gradual steady-state flux? What is the current state of thinking about the condition of enzymes in the cell wall?

DR. EVANS: Bob, you are getting a finger pointed at you by Stan here.

DR. CLELAND: The plant cell wall consists of a network of cellulose cross-linked by a series of hemicellulose covalently linked, hemicelluloses involving xyloglucans -- which are, in fact, probably hydrogen-bonded to the cellulose rather than covalently linked, although that is not entirely sure -- attached to arabinose galactans, attached to rhamnose galacturons, and then back again through arabinose galactans and xyloglucans to another cellulose. So they act as, essentially, a very heterogeneous crosslink between the cellulose.

The enzymes in the wall are very heterogeneous. The problem is, it is very difficult to obtain plant cell walls that are really completely and clearly free of cytoplasmic contamination. Actually, Bob Bandurski did some work on that a number of years ago and suggested there are probably anywhere from 10 to 50 different enzymes, at the minimum, in the walls. They tend to be in a state of a certain amount of flux. If you inhibit protein synthesis, the levels of some of these things turn over very rapidly, others persist for 24 hours unaffected.

DR. KRETSINGER: So a half-life of over six hours to six days?

DR. CLELAND: Yes. Six days is probably a little long. I would say that the half-life is probably more in the one-hour to 24-hour range in the cell walls. But it may be longer than that.

DR. KRETSINGER: And the sorts of thicknesses we are talking about are 100 to 2,000 angstroms?

DR. CLELAND: Generally, the smallest walls are probably about five microns, and they go from there up to maybe 100 microns, in growing walls.

DR. KRETSINGER: So there could be a very gradual steady flux?

DR. CLELAND: There could be.

DR. KRETSINGER: Any idea of the porosity?

DR. CLELAND: There are two conflicting papers that have been published in the last year or so, one of which says that the holes in the walls will exclude anything with a molecular weight above 14,000, and the other one -- which I happen to believe -- says that in fact that is too small, that in fact it really depends on molecular shape and how many holes there are and the distribution. Proteins up to 100,000 can undoubtedly get into at least part of the wall, if not into everyplace in the wall. But there are large areas in which proteins probably have difficulty getting in.

DR. HEPLER: Bob, you said that the wall might be from five microns. Don't you think that is a bit on the thick side?

DR. CLELAND: Maybe. What is it, one micron?

DR. HEPLER: Or maybe even a half a micron, but up to very, very thick.

DR. BANDURSKI: You can also get antibodies into a wall. Don Nivens has now got a specific antibody which stops growth.

DR. KRETSINGER: So one has lots of core diameters in there of 10 or 20, 25, 30 angstroms. A protein that big can get through.

DR. CLELAND: Yes, probably.

DR. BANDURSKI: Ninety percent of the chemistry in the world is done exterior to the cell membrane, because it is done by plants. Everything you are looking at has been done extracellularly by enzymes that are out there in the wall.

DR. KRETSINGER: I guess it is obvious, but it is probably worth emphasizing this. We all think of chemistry in a bucket, and approximation about activities and concentrations and so, where we have very, very small surfaces relative to volumes. When you are in a situation like this, where you have incredibly large surfaces relative to small volumes, the first 10 or 15 angstroms on any surface, a polyglycan surface, just isn't free solution. What we traditionally think of as concentrations, or even activities, are sort of completely off the wall when you are just dominated by the surfaces.

DR. CLELAND: Well, this is certainly true. This has been part of the problem, for example, in even just determining what the pH of the cell wall solution is. You can measure pH electrodes right up to the surface, as close as you can, but that is clearly not the same as potential in the wall, and there are domains within the wall. You can be half a pH unit one way or another, and -- I am sure the same thing is true with calcium and true with any other ion.

DR. BANDURSKI: This is partly what I had in mind when I asked you about -- I was not aware of the minute volumes and the very dilute solutions.

But, nonetheless, if you are talking here about a glycan and a gluconase localized in the wall -- and let's say it is amylase-like, with a low affinity for calcium -- it might be hard to put in so little of your chromogen that you would not affect the measurement. I don't know what kinds of concentrations we are actually talking about. But that is what I had in mind, really.

DR. ROUX: Dick Keefe is our next group leader.

DR. KEEFE: We will keep this fairly short. That was a very short hour for any kind of a lengthy discussion. Our group was John Horowitz, Peter Reed, Gordon Templeton, and Guy Thompson, and myself.

We spent almost the entire time period talking about the potential, anyway, in animal systems for alterations in nerve and muscle function, as variations in extracellular calcium levels occurred. Over the long term, long duration adaptation, I think we agreed that we probably would not expect much of an impact, once adaptation had occurred. But during the adaptational period, we felt there was some potential for some disturbance in both nerve and muscle function, on an individual basis. We talked briefly about some of the blood cells and the role that changes in extracellular calcium might play in the whole organism.

Gordon, you mentioned the model system that you have worked on with muscle, and it struck me that that has not come up at this particular symposium. Would you just briefly give a quick rundown on the changes you find in null gravity simulation? I don't mean to put you on the spot, but --

DR. TEMPLETON: I got into this about a year-and-a-half ago. The major objective was to take Emily Holton's model, which she had used to test its validity as a weightlessness model for bone, and see if it would be valid for skeletal muscle. We started out measuring mechanical properties. The most striking finding there was that we found that the soleus muscle speeded up; the contraction time and relaxation time decreased.

We then got into both histochemical and biochemical studies. The histochemistry, I will summarize it by saying that we really saw denervation type changes in both the gastrocnemius and the soleus muscles.

DR. THOMPSON: I think you have to explain exactly what the experiment was.

DR. TEMPLETON: It is pretty simple. We just take the hind ends of rats and lift them off the ground, so the rear limbs are non-load-bearing. They are not touching the ground. They are allowed to run around on their front paws. We have kept them up for almost six weeks. Typically, we have looked at one-week, two-week, and four-week periods, with one interesting result. Looking at the histochemistry of the soleus, looking at the myosin isozymes, we found that the fast-twitch component of the soleus doesn't change significantly, even over four weeks.

DR. HOLTON: Why don't you tell these people what the soleus is?

DR. TEMPLETON: The soleus is a slow-twitch muscle, anti-gravity, load-bearing. But its slow-twitch component decreased with suspension.

DR. TSIEN: Would it be different if you just immobilized? Is this anything other than atrophy?

DR. TEMPLETON: Well, that is one thing we want to look at. We just haven't gotten around to doing it, mainly because most of the literature has dealt with complete immobilization, putting them in casts and so forth. We would like to do that, just to have some data to compare, to make some comparisons between the Holton model and the limb immobilization. But we just haven't done it yet. There are so many different directions we can go. We are looking at recovery. We would like to get into the business of exercise, either pre-training or post-training, to see how it affects suspension.

DR. ROSS: I would like to put in a plug for that kind of work. In the space environment, especially, you have the added thing with the vestibular system. The vestibular system regulates the anti-gravity muscles. I think that is an exciting thing to be looking at, the neuromuscular interactions and what is going on. That is very important.

DR. KEEFE: We did talk about the possibility of some potential experiments so far as spaceflight is concerned. We felt one of the things that was lacking in all of the previous studies, particularly dealing with calcium, is an adequately done metabolic study of space-flown animals, particularly with respect to calcium and calcium balance. We felt this was something that would be worthwhile -- perhaps, as John suggested, a short-term, seven-day or less, but supersensitive type assay, something that can be done in a short-term time -- we have heard some examples just recently, in the last half-hour to an hour -- and then the potential for longer term exposure, longer than seven-day, but with an adequate control in terms of total metabolic study of calcium and its turnover in the organism. It doesn't have to be restricted to calcium. Then, finally, one I would toss in on top of that -- we did not get around to discussing it -- was the concept of a multiple generation series, where, instead of looking at a seven- to 28-day time span in the life span of an organism, we really need to consider some multi-generational capabilities in terms of studying all of these systems.

Any of the members of the group want to contribute anything more?

DR. ROUX: And last but not least, Carl Leopold.

DR. LEOPOLD: Our group consisted of Cormier, Jaffe, Krikorian, and Barbara Pickard.

We discussed three questions. We talked first about innovative materials that we could use for experiments on interactions between calcium and gravity. The second question we talked about was some possible experiments that could utilize ionophores or calcium-measuring dyes. The third question we asked was about possible specific gravity calcium experiments. Let me go through each of those. Several of the things will nearly repeat things that we have heard already.

Apropos of the first question about experimental materials that might be used to look at gravity calcium items, it would be nice if we could have a material that one could observe in the living cell and watch processes that were regulated by calcium. The suggestion by Dr. Cormier was that we could use the coelenterate Renilla, which is the sea pansy, which has the characteristic of having rings of bioluminescence, and the luminescence is apparently altered by a calcium system. So an initial set of experiments might be tried to see whether gravity forces applied to this sea pansy would alter the luminescence pattern. If this were true, if this would work out, it might be a very interesting material, and certainly very convenient, being in sea water and as mobile as it is.

The second type of material that we talked about was nonphotosynthetic cells that were good geosensors, and we talked about root caps. An innovative suggestion that was made, which I liked very much, was the use of the root caps from water hyacinth. These are quite easily pulled off, I understand. In my laboratory we have tried very hard, pulling off the root caps of things like corn and Vicia faba, and it is lots of work and you get very few good root caps. So I thought that the use of the water hyacinth was very attractive. Also, of course, one might be able to isolate individual cells from such a geosensitive tissue. That would appear to be another good possibility for gravity calcium experiments.

The classic material for studying gravity, the rhizoids of Nitella and Chara, were brought up again. These, of course, are so nice because they are so transparent. They are very small, but at least you can watch the inside of the cell without any complicated apparatus, other than a microscope.

Dr. Jaffe suggested the possible expanded use of mutants that have altered capabilities for responding to gravity. This is an area that has had very little attention. There are half-a-dozen papers in the literature on mutants which show differences in gravity responses. But I think that that is quite right; it would be a very interesting area to exploit.

Then, of course, there are the plant materials that become georesponding when they experience light. My experience with light-sensitive corn has not been very promising, but I think that there are still good possibilities of using a light-activated geosystem for this kind of a study.

Still in the area of how to do experiments on gravity and geo, we talked about the possibility of using in vivo systems that would reflect changes in calcium performance inside the cell. One of these that we talked about, of course, was the bioluminescence. That was particularly centered around the Renilla experiment. But in addition to that, it is, of course, very likely that cytoplasmic streaming has a very close tie to the free calcium that is available in the cytoplasm. So streaming itself could be an indicator that could tell us something about calcium changes. More recently, the discovery of a linkage of callose formation with gravity tropism offers some promise of watching things in the intact cell, too.

The second question was what we could do about ionophores, and looking at possible calcium concentrations with stains. I guess that none of us had very hopeful ideas about how we could use ionophores. I think we are probably all going to go home and try some. But we need to be careful about the interpretation of what happens with A-23. I think that Dr. Reed gave us a good sensible warning.

The third question was about the possible specific experiments on interactions of gravity and calcium. One of the most complicating things about looking at this in plants is that we presume that the cytoplasm is the interesting area, where a calcium change might be relevant to biological control, and the poor cytoplasm is stuck in between the cell wall, with simply enormous calcium concentrations. There are some data that have been published, if I remember correctly, with radioactive calcium where they have shown that millimolar quantities of calcium are bound in the wall. These surely must be in equilibrium with the solution.

Then, on the other side, we have the tonoplast, with often enormous amounts of calcium in it. You remember the pictures of even calcium oxalate crystals. Then we have the dynamic situation of mitochondria and chloroplasts which can pump the calcium in or out with their own signal systems. So it is a very dynamic system, and to be looking at even the concentration in the cytoplasm, much less the orientation of calcium, say, on tbe plasmalemma is a challenge.

We discussed the possibility of whether redistribution of calcium might be the only way of obtaining such an interaction with gravity and calcium. It was pointed out that redistribution, or a change in distribution of the calcium-binding protein, could also be of interest in this regard. We talked also about the possibility of obtaining gravity-induced calcium redistribution through the movement of the amyloplast itself. We have recently shown that calcium was present in that amyloplast.

So, except for some duplication of suggestions that other groups have made, that is the best I can recollect.

DR. KRETSINGER: You spoke of the removal of root tips. Is the root tip, after its removal, sensitive to a gravity gradient? How does it then respond?

DR. LEOPOLD: I am not sure that I can remember the exact experiment, but Pilet did experiments where he pulled off the root caps of corn roots -- the root cap has the sensing cells in it, you know; that is where the amyloplasts are that fall --

DR. EVANS: You should explain the difference between a root tip and a root cap.

DR. LEOPOLD: The root cap is a cluster of cells that covers the actual growing point of the root. So the root has a large meristematic region in here, and then there is an actual cap layer, in fact, of specialized cells that do two kinds of things that we know of. One is that they are loaded with amyloplasts, so a change in orientation with respect to gravity causes a redistribution of these in each cell.

The second thing that the root cap does is to secrete actively slime. Actually, they are oligosaccharides. It is a complicated carbohydrate lubricant, in fact, which serves to ease the movement of the root tip through the soil.

So this is a root cap, containing these amyloplasts, and it can be removed. Pilet was able actually to pull it off, and, if I remember correctly, he gave it then a horizontal exposure and then pushed it back onto another root tip, this part. The root tip then showed a geocurvature. So the root cap, I think, can independently sense gravity.

DR. KRETSINGER: But to assay it, you have to put it back onto a tip?

DR. LEOPOLD: Yes. We don't know what the signal is that the root cap gives to the tip of the root. We don't know whether it is a hormone or whether it is calcium. We have no idea.

DR. WATTERSON: Does anybody know anything about motile plants, like Chlamydomonas, and what happens to them in hyper- or hypo-gravity?

DR. KEEFE: There have been so many, I don't know.

DR. KRIKORIAN: Chlorella has been, only concerned with photosynthetic activity.

DR. WATTERSON: And that is normal?

DR. KRIKORIAN: Well, as Thora said, in some cases yes, in other cases no.

DR. ROUX: This has been done exclusively by the Russians?

DR. KRIKORIAN: Yes, Soviet stuff.

DR. LEOPOLD: I think with the new orientation toward possible interactions of gravity and calcium, it is worthwhile to ask ourselves what are kinds of materials that could be specifically exploited in looking at this kind of possible interaction, or to work out more quantitative details. I think that we are going to have to be innovative in finding materials that are easily applicable to this kind of question.

DR. ROUX: That ends our discussion period this afternoon, except that I would like to make this comment. We are not setting aside a formal period where people can just generally ask questions to each other about things that they have heard in the talks. We have had some really outstanding talks over the last day-and-a-half. I have been very impressed with the quality of them. In almost every case, we haven't had enough time to really ask all the questions we were interested in. I know it is getting late, and maybe tomorrow might be a more appropriate time for this, but I assure you that there is absolutely no reason why, within the context of the structure of this workshop, we can't have a period of time when we can have just open questions about any of the talks or any of the material in the talks. I think maybe we can kind of plan on this tomorrow, since it has been a long day today.

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DR. ROUX: I guess we will get started with Carl Leopold.

DR. LEOPOLD: We are going to spend our time talking about two models, and I know it is easy to shoot them down, but I think it is better to start with models that at least you can work from and do some experiments on. So, we are setting up a target here for you to shoot at.

We are going to assume that there must be at least two gravity sensing systems in plant cells, and we have named them the auxin transport geotropism for the case of young tissues like coleoptiles and root tips and the hormone release geotropism as in the case of the pulvinus in grasses where these are old cells.

We have, in the model of the first case, the first step being the reorientation of amyloplasts. So, as the amyloplasts fall within the cell and approach then the new lower side of the cell they induce a lateral transport of auxin. You can imagine that this is a cell in the middle of either the root or the coleoptile. Now the amyloplast moves to the base and activates an auxin-calcium symport. We are not sure that calcium and auxin move together -- that it is a symport for these two, but Rollo Dela Fuente is utilizing a new system that was established by Reiner Hertel, who has isolated vesicles from zucchini squash stems which perform auxin transport in vitro. So, using radioactive calcium and radioactive auxin, Rollo now is in the process of establishing whether you can tell that calcium is traveling on a symport with the auxin in vitro.

So, we are assuming that as the lateral transport of auxin occurs, activated by the amyloplasts, there is a synchronous movement of calcium in the opposite direction.

DR. BIKLE: Just for those of us who are not tuned in too much to plant physiology, I take it that the amyloplast is a subcellular organelle which contains auxin?

DR. LEOPOLD: No, it is an organelle that is very much like a chloroplast but is filled with huge starch grains. It has a little endoplasmic reticulum and --

DR. BIKLE: The endoplasm inside has its own ER?

DR. LEOPOLD: It has its own ER and it has a zeta potential. That is, the outer surface of the amyloplast carries a negative charge field. We are going to assume that as this charged field approaches the plasmalemma, or something that is involved in the transport of calcium and auxin in the opposite directions, that the change in the electric density in the region of the amyloplast would activate this membrane. That is a big if. We don't know this.

DR. WATTERSON: Are auxin and calcium sources inside the same cell?

DR. LEOPOLD: Yes, in these young tissues there is plenty of auxin, and yes, and the calcium is everywhere. So, there is lots of calcium around, particularly in the cell wall.

DR. WATTERSON: Do you know what glycosins are? There was a report on that.

DR. LEOPOLD: No.

DR. WATTERSON: Cell biology people might know more about it. I was wondering if there is any type of subcellular structure in these type tissues where you would have metabolic machinery; you know, in the fatty acid system you have a plasmic and a mitochrondrial beta oxidation system.

DR. HEPLER: Do you mean glyoxysome?

DR. WATTERSON: Yes. Wasn't there a carbohydrate metabolism on --

DR. LEOPOLD: Lipid transformation.

DR. WATTERSON: There is no carbohydrate metabolism.

DR. HEPLER: Oh, yes, there is lots of carbohydrate metabolism.

DR. LEOPOLD: But please remember that the recent evidence now at the ultrastructural level indicates that the amyloplast is not just a big bloody stone, just a dead weight, but instead it is capable of doing a lot of, presumably a lot of synthetic biochemical transformation.

DR. WATTERSON: I was just wondering about compartmentalization of the calcium effects.

DR. LEOPOLD: We are going to assume for this model that the calcium, the principal calcium here is moving from the cell walls on into the cytoplasm.

Bob?

DR. CLELAND: You remember the old Browner experiment where they took sunflower stems, put them on their side at zero degrees where you would expect metabolic activity essentially to cease, then proceeded to put them back upright at zero degrees and leave them for anywhere from zero time to longer time to see if there was a memory in those cells of being on their side, and there definitely was, and it was almost an hour before that disappeared. How would you relate that to anything involving direct metabolic activity from your amyloplasts? I find that very hard to see.

DR. LEOPOLD: Okay, I think an escape from this might be that a change in the electric potential across the membrane might be preserved at the low temperature, and this could be a candidate for such a membrane, but let me go through the whole model.

DR. PICKARD: I think those experiments are not conclusive. I repeated all those extensively. I spent three years doing it, and they are shot to hell with problems. I don't think that the basic conclusions from those were right. The work was totally based on some mathematical assumptions. The receptor system is destroyed rapidly as a function of low temperature, and this was interfering with their measurements; their conclusions are invalid.

DR. CLELAND: I don't understand. Do you mean you are saying that if you put them on their side at zero degrees and then put them upright for, say, 10 minutes at zero degrees before you warm them up that they don't bend?

DR. PICKARD: That is extremely complicated kinetics. Their plants were responding even at zero or at 2 degrees in point of fact.

DR. LEOPOLD: Rather than diverting too much into the side exchange, let me tell you about the two models. Okay? Because I think that there are some interesting general possibilities between these two. So, you have an amyloplast lateral movement and an activation of an enzyme that secretes auxin acid from the inside to the outside, and calcium comes in. Let us assume that that is going to go together, either on a symplast or two different transport sites, and that then you end up with a differential growth because of the accumulation of auxin on the lower side, and the accumulation of calcium on the upper side, the former being stimulatory to growth, the lateral being inhibitory to growth. Okay? And so, now leaving that just for a minute, I would like to then contrast that with our second model, and that is a hormone-release geotropism in which the amyloplast falls now in maturing cells which are vacuolated. The amyloplast falls and carries with it tonoplasts.

So, the amyloplast falls, carrying with it, dragging with it, we will say, for the moment, tonoplasts, and the tonoplasts approaching the plasma membrane cause a depolarization and a change in the permeability of the plasma membrane. If you have these events calcium then can leak in, and the calcium entry and resulting attachment to binding proteins would activate in our model, enzymes that would release auxin principally from the inositol form or gibberellin from a glucoside form.

SPEAKER: ... or from the peptide derivative.

DR. LEOPOLD: From the peptide derivative? Wow, that is a bigger job. All right. So we can have preferential synthesis of these two hormones on cells on the lower side. These enzymes will loosen the cell wall and you get growth, and you will ask right away why should this occur differentially on the lower side and not on the upper side? You remember from Peter's pictures that these cells in the pulvinus have different anatomy even between the upper and let us say toward the center. The radial and centripetal side and the centrifugal side are physiologically and structurally different and they presumably have different enzymes on them. So, if the amyloplasts dragged some tonoplasts to the centripetal side then it would presumably not activate such enzymes, but in moving to the centrifugal side, it would.

Now, those are very hypothetical things, but in each case you will see these two models. Calcium plays a component role but not in sensing. It is in the transduction of the signal from gravity and in the activation of enzymes that calcium changes would result in differential growth. In this case the calcium would presumably be an inherent part of the auxin transport system, and Rollo Dela Fuente's experiments strongly indicate that the redistribution of calcium occurred. Here the calcium is playing a passive role as far as the sensing mechanism is concerned. One more thing that I would like to say before opening it up for everybody to jump on it, is that this transport geotropism would depend on an amplification system; that is, one cell would make precious little difference in the redistribution of auxin down, calcium up, but if you have slightly differential frequency of your activated symport side on the lower side here, as you multiply this with more cells you would get presumably an exponential increase in the polar redistribution auxin down, calcium up. Rollo Dela Fuente's early experiments of 10 years ago indicate that that is in fact the case; that is, that the redistribution of auxin is a function of the cell number. So, you can have a very small polar difference between two sides of each cell, and if you multiply this by a whole string of cells, then you can get a large polar effect.

So, fire away.

DR. ROUX: One aspect of this that intrigues me is the possibility of doing specific experiments and testing to test the hypothesis. Certain points here which are very testable. I would like to ask Bob about the enzymes that would be involved in the release of IAA: specifically, what are their names, and have they been isolated? Can they be tested for their calcium dependence or how they are regulated?

DR. BANDURSKI: The only enzyme so far that I have isolated, and I mean gotten into a crude extract, is the enzyme that hydrolyzes IAA inositol. It has a high degree of stereo specificity that hydrolyzes one of the equatorial esters. It occurs down in the tip of the plant, but it is only one, and as Peter has already indicated in the case of other plants where you have a peptide conjugate, for example, IAA aspartase or something we know nothing about; either its in vivo biosynthesis or its in vivo hydrolysis. So the only enzymes that we know about are really those that make the IA myoinositol conjugates and hydrolyze them, but we don't know anything -- they are too crude --

DR. LEOPOLD: Bob, what about gibberellin releasing enzymes? Can you help us on that?

DR. BANDURSKI: Yes, Semner has one, Lear two, and there is, also, cytokinin hydrolyzing one. So, yes, those three are diazo 21.

DR. ROUX: I think maybe a general background note for those of you who are not plant physiologists is that a large percent of the hormones in plants are present as inactive conjugates and that frequently when you get a rise in active hormone in a plant tissue it is because an inactive form has been activated. An active form has been released from a conjugate by some metabolic process. Is that correct, Bob?

DR. BANDURSKI: I think that is very true.

DR. ROUX: As of yet we know very little about the enzymes involved in that very critical step and that would be really crucial if we wanted to test this hypothesis. Is that correct, Bob?

DR. BANDURSKI: Oh, sure, it is doable.

DR. THOMPSON: Would this model call for an increasing level of extracellular ionic calcium across the tissue?

DR. LEOPOLD: Yes, I would think that would be consistent.

DR. THOMPSON: Are there plans for testing that?

DR. LEOPOLD: Bob Slocum's experiments and Stan Roux' experiments have really indicated that there is an accumulation on the upper side, but that is not a gradient.

DR. THOMPSON: I am thinking that a good potential sensor for calcium in its ionic form apart from these dyes and so forth would be the state of the membrane fluidity. We have done this in simple cells. If you could very quickly fix with glutaraldehyde, shall we say a section of the stem after chilling the tissue down to differing degrees, say, at 15 degrees, 12 degrees, 10 degrees, etc., and you could find a point where there would be an incipient phase separation of the lipids, it might be sensitively affected by the levels of external ionic calcium, so that you might see in the cells on one side of the tissue a greater phase separation at a certain temperature than the phase separation on the opposite side of that same stem.

DR. LEOPOLD: How would you look for the phase separation?

DR. THOMPSON: You would look for that by freeze fracture EM. You might or might not be able to see it. In some tissues you can; in some other tissues it is not so easy, but it would be a potential way. Not only would you be able to see a very short-term effect on membranes, but membrane lipids have a kind of memory in the sense that again, in some tissues that have been studied, they would adapt over a period of hours to a higher or lower level of cation, so that you might be able to see both the short-term effect and a long-term adaptive effect which would give you some of the prehistory of that particular tissue over a period of time. So that might be one good way in space, for example, of taking quick samples after chilling to differing degrees and looking at them later by freeze fracture.

DR. TSIEN: Do you think that after you saw a difference in freeze fracture appearance, you would feel confident to say that was due to calcium?

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DR. THOMPSON: You would have to do lots of controlled experiments obviously back here on the ground. Of course, the fluidity is affected by many other things, and you would even have to check auxin, etc.

DR. LEOPOLD: NMR would be another really nice way of asking these questions because the fluidity of the membrane phospholipids can be tested with NMR and you don't have to mess it up. I mean you leave the tissue intact.

DR. THOMPSON: On what membrane, that is the question.

DR. LEOPOLD: No, but you could perhaps cut your tissues between the upper and lower sides and compare them.

DR. BIKLE: A way with the same advantages of NMR, if you could get your separation of your membrane, is with fluorescence polarization studies which would be equally very simple to do. You don't have the advantage with either NMR or polarization techniques of being able to fix the tissue and look later. You would have to study the situation as it would exist then, in other words preparing your membranes in a reasonably fresh situation and then do your studies.

DR. THOMPSON: That is the problem in the plant. You have an almost insuperable problem even preparing homogeneous membranes unfortunately.

DR. BIKLE: NMR technique and fluorescent polarization would require the ability to prepare membranes that are reasonably homogeneous from the relevant areas.

DR. LEOPOLD: But it seems to me the weakest link in this model one is whether calcium can move -- whether you can relate the calcium import to the auxin export or whether this would be a change in the polarization of the membrane quite independent of or consequent to the auxin transport. Would anybody like to work on that?

Peter?

DR. HEPLER: Yes, I will probably remind you of an experiment you have known for a long time, since I was hardly born when this came about. This is Beatrice Sweeney and Thimann in 1937, who showed that the very most rapid response of auxin was an immediate increase in the rate of cytoplasmic streaming. It went up for about 15 or 20 minutes and then came down.

Now, to the extent that cytoplasmic streaming is sensitive to the levels of calcium, again you have to be careful there.

DR. LEOPOLD: Has it ever been shown, the calcium?

DR. HEPLER: Yes, in Nitella. Now we have pretty good evidence that the increase in calcium concentration shunts down cytoplasmic streaming.

DR. LEOPOLD: That is neat.

DR. HEPLER: So, if you could argue from that, then you would say that the auxin increase has caused a decrease in cytoplasmic free calcium which is allowing for the stimulation, by whatever mechanism, of the rate of cytoplasmic streaming.

DR. LEOPOLD: It would be something you could really look at.

DR. HEPLER: What I am saying, I guess is that perhaps there is a little thread of evidence which would suggest that auxin and calcium are cooperatively changing, perhaps in the opposite direction. As auxin is going in the calcium may be going out.

DR. LEOPOLD: Extremely crucial to this is that streaming does occur in the geosensing cells in the root cap. Of course, it is, also, very common --

DR. HEPLER: These were done in coleoptiles.

DR. LEOPOLD: Coleoptiles. But the root cap is such an attractive geosensor.

DR. HELPER: But anyway streaming is, again, something that has not been looked at very much but could be.

DR. LEOPOLD: That is very nice.

DR. EVANS: Carl, as far as I know the evidence is that streaming doesn't play any role in auxin polar transport. I don't know about gravitropism.

DR. LEOPOLD: That is right, but Peter's point was simply that you could use streaming as as overall index of changes in auxin and calcium.

DR. EVANS: Another point is that I wonder quantitatively whether a simple calcium-auxin symport model is reasonable because you are dealing with very low, quantitatively auxin redistribution, and presumably the amount of calcium moving has got to be lots greater than that if you are going to cause inhibition of growth on the top. You would have to have a probably unbelievable stoichiometry moving --

DR. LEOPOLD: I think that is a very weak point of the symport idea. Bob Slocum, could you comment on how much calcium accumulates on the upper side, say, of a coleoptile?

DR. SLOCUM: From my data it is not really possible to quantitate. That is a qualitative localization technique, but I know the papers of Goswami and Audus and I believe they did not find more than two or three times as much calcium on the top as on the bottom following calcium 45. It is not a real large change.

DR. EVANS: But still in terms of total calcium moved, it would have to be much greater than the total amount of auxin.

DR. BIKLE: Carl, aren't your energetics wrong? When I see a cell pumping an anion in and a cation out I start to think that there is no such mechanism.

DR. LEOPOLD: I think this is a very serious problem with the symport idea and only Black and Andlelav and myself assume that there is a proton pump involved. There is lots of evidence that auxin stimulates the proton secretion. Whether it is associated with this or whether it is simply going along on a separate --

DR. BIKLE: I don't think you have to postulate a symport anyway.

DR. ROUX: No, two separate mechanisms.

DR. BIKLE: Yes, I mean you don't need it.

DR. LEOPOLD: I looked at that as a minor hurdle. I just submitted a paper to Planta suggesting this, and I cannot even remember why we wanted to suggest that there was a symport. I am not sure.

DR. ROUX: Just to raise a general problem and that is just moving calcium either in or out of the cell does not explain how does it get into the walls on the upper side. What is the method of apoplastic or extracellular movement of calcium? What kind of mechanism could you invoke that would move calcium extracellularly to one side? Your model would address itself to how do you get calcium in and out of the cell, but it would not address the question of how do you get it to migrate extracellularly to the upper side, and that is a very puzzling question to me.

DR. LEOPOLD: Yes, that is a tough one, and it may be that if there is a preferential import of calcium on one side that, say, calcium ATPase is on the plasmalemma, I don't know. Some people think they are.

There might be a non-polar export in other direction and then it would be picked up by the next cell and do the same thing again, and once you start repeating that cell-to-cell you can imagine that you would get a very substantial accumulation after crossing quite a number of cells to the upper side. I am still not answering the question of how the calcium may go out of the cell. If it is coming in here, then how do you conceive of it getting out? I have not really answered you.

DR. CLELAND: It would seem to me that one piece of evidence that would be essential for such a study is the simple question does auxin affect the calcium going in and out of cells. That is easy enough to measure, and I would raise the question is there any, are there any published data on this?

DR. DELA FUENTE: I have presented papers at the last two meetings of the plant physiology group which show that the amount of calcium that you can detect at the apical end is promoted by the presence of auxin moving in the other direction, basipetally. If you reduce the amount of auxin in the tissue, the amount of calcium transported is less.

DR. CLELAND: Transport is one thing, but one could simply measure the rate of calcium uptake into individual cells in response to auxin or to the entry of auxin or in a pulse chase type of an experiment one can measure calcium flux in response to auxin leaving the tissue. Does that change the uptake or efflux of calcium? It seems to me rather incredible that to the best of my knowledge there is no public data.

DR. LEOPOLD: Rollo's present experiments will, I think bear on that because he is going to use calcium 45 and carbon labeled IAA and ask about the simultaneous movement across the vesicle membranes, the zucchini squash. So, I hope we will have some evidence on that very soon, I mean next month.

DR. ROUX: I think Bob appreciates the fact that that would be a difficult experiment, the one he suggested because, for example, if you wanted to do that in protoplasts, would you really believe it? How would you stimulate with auxin, from the outside, and would you believe there would be auxin binding sites on the outside of the cell? But we should talk about it.

DR. CLELAND: I don't think it is as bad as that.

DR. ROUX: Bob Kretsinger, could you come up here, please?

DR. KRETSINGER: The members of our discussion group were Mike Bennett, John Horowitz, Gordon Templeton and Roger Tsien. I am Bob Kretsinger. We posed several questions. The one we pursued for most of the while was one of not so much technique of measurement of free calcium ion but some of the underlying concepts and reference points. It turned out that rather unwittingly I stepped into a review article that Roger had recently prepared and perhaps the best way to elicit comments from him is to slightly misrepresent what he said, but I will try.

As you know the activity coefficient of calcium is significantly different from one, and there is some question about what the expression of free calcium ion means, and how valid is the approximation, and I think the important point is that one can define activity with reference to any standard condition that one wishes, and there is nothing particularly sacred about 20 degrees in pure water, and it may not be the most appropriate reference point for physiologists. There is a significant change in activity going from zero salt to say, 150 millimolar salt. To go from 150 millimolar salt which might be an appropriate reference point for which one can define activities in non-ocean systems to go on up to, say, something like 400 millimolar potassium chloride to approximate seawater represents a relatively small additional increment in change in the activity coefficient. So, it is of obvious importance that in any determination of calcium concentration or of binding constant it be stated, what the ionic environment is.

We then questioned whether or not it would be appropriate to urge or recommend that people who are working with such systems adopt one or a small number of reference conditions, for instance, ask people who are doing such determinations to try to work at one set of conditions, say, 150 millimolar potassium chloride and pH 7, or 400 millimolar. Whether anyone has the authority to make such a proposal, of course, is also a question, but, again, I think the relevant point is that once one is above 100 millimolar going on up to 400 millimolar the incremental change is relatively small, relatively,to going down to zero millimolar ionic environment.

Then the sort of the next question one might consider in the series of considerations is the extension of these in vitro definitions and determinations to in vivo systems that we are really interested in. A point that I have referred to in discussions and one I have wondered a great deal about is the extent to which the very, very large surface-to-volume ratio within cells is relevant. That is, in the test tube there is so little surface, whereas if we are within a cell right now and there is a 15 angstrom layer along the surface of the cell, where there are significant electrostatic fields, where there are a significant number of potential calcium ligands, so that the calcium ion is not just your standard hex or heptahydrate ion, I wonder whether the surface-to-volume rates matter. Again I think that probably the consensus is that it is an effect that one can ignore in the following sense, that as long as some component of the volume out here in the center of the room is reasonably well removed from the boundaries and the system does equilibrate, that still it has equilibrated with the environment that is far enough away from the wall to be reasonably approximated by the in vitro determination, one of the various binding constants or calibrations that one is doing with an electrode or a dye or whatnot.

So, given that proviso, then that probably is an adequate definition and an adequate reference point. Now, the perhaps more important consideration then is yes, the hexa-aquacalcium ion out here in the center of the cell compartment is in equilibrium with the calcium ions that are near the boundary, whatever their state is, penta-aqua with a hydroxyl ion providing the sixth coordinate on average, that as long as it is equilibrated with the ion in the center of the room, our reference determination with the microelectrode or dye or so on is adequate.

Very frequently of course, we don't care about the activity of the calcium ion per se. What we care about is the combination of the activity of the calcium ion, plus the entity, the protein with which it is interacting. There one has to make the assumption that not only is the apparent activity, the first assumption that I just described about the calcium ion and the wall effect, but then a second assumption, the second component of the system is the calcium modulated protein, and can we assume that the binding constant of calmodulin out in the center of the room is the same as the binding constant of calmodulin that is up against the wall. That seems to be a tough one in the sense that there is just now a priori way of being able to calculate that. I mean to really drive home our humility, if I give anyone in this room or the world the coordinates of the oxygen atoms and the entire protein wrapped around a calcium ion, they cannot calculate to within two decades what the binding constant is. They have precedents that give them good guesses, but you know we are not near being able to make that ab initio calculation, and this is even a step more difficult. So then one again comes back, is there some experimental normalization for this, and I think the suggestion, the consensus would be that the best we can do, and it might be adequate at this time, is to determine the binding constant of calmodulin, not only in a defined and stated and adhered to potassium chloride concentration, etc., but that it might be an adequate, if not perfect approximation to determine that binding constant *in vitro* in the presence of a fragmented membrane preparation. Then one could see whether, relative to the dye, relative to the electrode *in vitro*, the presence of an adequate surface of membranes would shift the binding constant. That seems to be the best idea we have at this time of how to approach that problem experimentally.

A final problem then that I posed was how valid was the assumption of equilibration in the previous example, that is in dynamic systems. We often see these pulses of calcium ions, as suggested by aquorin emissions or arsinazo dye or whatever, that the actual pulse of calcium ion occurs over a rather brief period of time and often times rather brief period of time relative to the subsequent series of physiological events that we want to follow. Given that brief period of time in which we see the pulse and the rather convoluted nature of some of these membrane spaces, is the assumption that anything approaching equilibrium has occurred over that time a valid one? I don't think that we really reached a consensus on that because the calculations really have not been done in the first place to get a reasonable approximation of cross sections and of length over which the diffusion must take place. A second reason we were unable to answer this question is this. We have some idea of what the handbook diffusion constants are for the aqua calcium ion. But in this sort of space where a significant fraction of cross sectional volume through which the calcium ion has to diffuse is near the wall, whether the apparent diffusion constant is going to be decreased by a factor of 2 or a factor of 10 seems to be an open question. But, again, I think the sorts of experiments that we have available to us now indicate that for many situations the assumption of rapid equilibration is probably going to be a relatively safe one.

Those in the Committee who feel that I have not properly stated the case or the divergent views obviously should speak up. Roger, you have done a lot of thinking about this. I think these were the ideas that we addressed in greatest detail.

Are there any other comments or questions?

DR. BIKLE: Actually, I do have a question that you addressed, and this is a question I asked Rasmussen when he implied in one of his cartoons that there was a change in calcium binding to calmodulin with its association with its particular, in this case, phosphodiesterase, but we could take a membrane bound, calmodulin binding protein of the membrane to go along with your formulation. Is there any evidence that such a change in calcium association to calmodulin takes place under those conditions?

DR. KRETSINGER: I think that one can infer that there are some changes in the conformation of calmodulin upon binding with some of these targets; sort of in the big picture view of the ongoing research on calmodulin, obviously the first thing to attack is to try to characterize calmodulin itself, and many of us are in the process of doing that. But that in itself is not terribly interesting. What we are interested in as biologists is the next class of questions and that is the interaction of these various targets, and I don't think the first one has been adequately resolved to allow us to really get into the second one, but at the structural level what I and various people would like to do is try to crystallize a calmodulin target complex and look at that structure.

What spectroscopists would like to do is to take NMR, CDR spectra of the complex and see if it is simply additive. Are there deviations from additivity? What the thermodynamicists would like to do is to determine the binding constant of calcium to the isolated protein and then the apparent binding constant to the protein as complexed with calmodulin, and various classes of competition experiments of that sort. I think that none of us have crystals. That one is easily answered.

The preliminary sorts of NMR results I think indicate that much of the spectrum is accounted for by additivity, but there are identifiable non-additive components in the spectrum indicating a significant conformational change. I think where the numbers are better known is the case of troponin C, but I think the precedent is very good there. The apparent affinity for calcium of the whole troponin complex is about a decade higher than in the isolated TNC and one assumes it is the same nominal sites involved. I think that is, in very brief summary the state of the art there.

Roger, you had some further comments?

DR. TSIEN: I was going to mention the troponin data just the same, and if troponin can do it one expects other calcium binding proteins can, too. Also, just from thermodynamics, it seems to me calcium strongly affects the equilibrium binding of protein A for protein B, then probably the existence of protein B just by thermodynamics will change the affinity of calcium for protein A the way oxygen and acidity interact on hemoglobin. They just have to by thermodynamic linkage.

DR. KRETSINGER: Right. I said the apparent concept, Chockley's principle, you have driven it over this way. Now, there may be additional subtle mechanisms beyond that, but I don't think any of the data that we now have allows to distinguish them or go that additional step.

DR. BANDURSKI: One way to solve the difficult physical chemistry would be to look for a case where one can measure the activity of an enzyme in the cell and see whether or not the activity is, in fact, what we would predict from the measurement of ambient free calcium concentration. I wondered if there had been any attempt to measure flux, in any system where we know enough about the enzyme that we could do that sort of thing.

DR. KRETSINGER: I think that either Milt or Marty are much better qualified to address that. That is obviously one of the next generation of questions. Off the record, what would be your speculation, Milt?

DR. CORMIER: Those measurements are difficult. I mean they really haven't been done. Maybe, however, one of the systems that could be utilized is some of the calcium dependent bioluminescent ones.

DR. BANDURSKI: We could monitor the amount of light, steady state initiative rate as a function of what you think steady state free calcium is and whether they coincide at all.

DR. BANDURSKI: But *in vitro* bioluminescent proteins are strongly affected by all sorts of other modifiers like general anesthetics, temperature, magnesium, pH, heavy metals. They are affected by all such things, almost anything, well, not that bad, but plenty of things.

DR. KRETSINGER: Any other comments or questions?

Thank you.

DR. ROUX: Thank you, Bob.

Next report from Dick Keefe.

DR. KEEFE: My group this morning consisted of Peter Hepler, Emily Holton, David Spray and Guy Thompson. We had an extremely interesting morning, to say the least. After some initial discussion, a couple of little ideas on bone loss and marine mammals and size required of marine mammals and so on, Peter Hepler posed a question. For an organism to perceive gravity, does something have to move? If you look over here and you see amyloplasts and they are moving, obviously they are perceiving gravity. Most people thinking in animal systems think of statoliths, and the statoliths have to move or at least be tempted to try to move to produce the transduction.

We got into a little bit deeper discussion on this, a quite a bit deeper discussion. If you start to think about statoliths so far as vertebrates are concerned and other animal species, we deal with conscious perception of gravity versus perception of gravity by the individual cells. It is, I would argue, a different factor. You feel the gravitational field, and you can express that you feel the gravitational field, as opposed to the impact of a gravitational field upon an individual cell. Those are potentially two different sensing mechanisms in terms of gravity, and for our evolutionary history we have shown the development of this vestibular system, and we have become very dependent upon our nervous system to recognize what is up and down, but I would maintain that at the cellular level and at the tissue level the cells are perceiving something. They are perceiving gravity. You are just not communicating that into the central nervous system.

So, we got involved with do you have to have movement? If you have to have movement to perceive gravity at the cellular level, how could it be affected; how would you cause this transduction, and we got involved with the potential for lever arms, organelles attached to cytoskeletal elements which potentially would be anchored in the membranes. That then led to the consideration of is there sufficient difference in specific gravity in the density of the organelle versus the density of the cytosol to cause the shift to occur, to respond.

That led us to the concept of the size required in individual organisms for perception of gravity at this level. The concept would be that potentially an individual cell could recognize this if there is a lever arm mechanism and some organelle or distribution of organelles is acting to shift the pattern of stresses on membranes and therefore cause shifts in cell shape, potentially changes in cell size. If you get two or more cells put together, then you have got tissue that you can then start expressing that stress across and potentially amplify the effect. This leads me to consider the potential of development and the developmental pattern as cells undergo differentiation to form highly specific tissues. We are becoming more and more aware of the differences in connectivity patterns, gap junctions and so forth between these cells as they undergo development to subsequently at the point where the cell becomes pretty much locked in place. If you think about the forces that are impinging upon a cell at any one point, not only in terms of junctional complexes between adjacent cells and between basement membranes and connective tissue fibers and the extracellular space, those forces at the cellular level probably play a much greater role than the direct import of gravity upon the cell, certainly in terms of short-term actions on forces. The question would be over a long duration exposure to an altered gravity or the lack of a gravity might you suspect that there would be additional changes taking place.

We then considered some of the changes that would occur at the osteocyte level in bone as the organism would undergo changes in gravity, particularly in the cell itself, at the cell-to-calcium, if you will, or the cell-to-bone mineral interface and how might we expect changes to occur there. This led us into a discussion of gap junctions and the consideration of communication between cells within bone as a tissue for trying to transduce changes, to signal changes from one spot to another within the tissue so that one bone cell could let another bone cell know what was going on in there.

We did not get too far on that one. We got involved in a lot of other discussion from that, and one of the things was what if we were to look at bone cells or other cells in an organism being subjected to changes in gravitational fields? What organelles would we look for, what changes at the cellular level would we start looking for immediately? Where did we feel we would have changes that we could start searching for? One of them, that David Spray suggested was the gap junction, that we could look for changes in size, distribution, quantity of gap junctions between adjacent tissues; certainly this would be a very useful tool in terms of developing systems being exposed to altered gravitational fields. Guy suggested that we look at some lipids and changes in lipid membranes, and that led us to thinking about what organs in an animal that had been subjected to null gravity would we start looking at these changes in, from the standpoint that certain organs are going to be secondarily impacted by the organism's exposure to null gravity. For instance, the kidney is going to show changes because of cardiovascular differences, fluid electrolyte balance shifts. So, in the kidney we would be potentially looking at secondary changes as opposed to primary, and one of the potentials here is the liver, but that, too, would be secondarily affected. We talked about the potential for examining brain which seems to be a pretty slow, highly conservative type of tissue and also the potential for thymus where you have got a fairly well characterized system of cells and you can pretty well pin down what you are looking at.

We, also, thought it would be a very useful technique to examine calmodulin or calmodulin-like proteins distribution in different species as they are subjected to different gravity effects. For instance, this could be readily tested on a centrifuge showing some hypergravity action and asking across several species whether or not you see a common pattern of calmodulin changes or calmodulin protein-like changes. The same sort of thing could be done with membrane lipids. There was a slow discussion of a slam-dunk technique for preparing specimens and that led us finally to the conclusion that almost every one of the projects that we talked about showed a very definite need for manned operation. This meant it would be necessary to have a manned laboratory in order for sampling to take place under conditions of null gravity.

I sort of capsulized and summarized right straight through in case there was some discussion, and I have been watching the clock at the same time. For the members of the group, does that pretty much cover what we emphasized today?

Does anybody have any questions or comments?

DR. KRETSINGER: I was just curious. I cannot imagine why one would suspect that the concentration of calmodulin would be interesting, would be a significant parameter. Why do you propose that experiment?

DR. HEPLER: I think it was more the distribution of calmodulin.

DR. KRETSINGER: By distribution are you meaning tissue by tissue?

DR. HEPLER: Or within a cell, too.

DR. KRETSINGER: Organelles?

DR. HEPLER: Right.

DR. KRETSINGER: Why would one anticipate that that would be an interesting question to ask?

DR. HEPLER: Certainly it has been interesting to me that in the dividing cell calmodulin changes its distribution. Now, whether this has any functional significance or not is another question. It lights up the spindle poles and it is clearly related to the process of mitosis, at least from a structural point of view. What its function is we don't know.

DR. KRETSINGER: So, it was posed not as an indication that the quantity of calmodulin would change?

DR. HEPLER: No, I think --

DR. KRETSINGER: But as a reflection of where the targets, where the action was?

DR. HEPLER: Yes.

DR. KRETSINGER: I see, thank you.

DR. KEEFE: That, also, has the advantage of being an experiment that can be looked at on a ground-based situation first, to see whether or not there are changes as opposed to having to be a null gravity type proposal.

DR. BENNETT: This may have been discussed day before yesterday, when I wasn't here, but it came out this morning that in terms of the slow kind of cellular detectors that one might expect to exist, like the amyloplast, that if you tumble the preparation, the animal or plant never knows which way is up or alternatively down, and this, from a theoretical point of view would be equivalent to zero G conditions or very nearly the same as zero G conditions.

In terms of development of eggs, as far as teleosts are concerned there is a biological or natural experiment that is done all the time by pellagic species: they just spread their eggs out, and they are free floating, and I presume that they are under pretty turbulent conditions and are basically tumbled and the fish come out fine. Has that been done with amphibians?

DR. KEEFE: I am not aware of clinostating eggs of amphibians. In Fundulus there have been a fair number of studies, including clinostating Fundulus eggs for the entire developmental period and for selected times during development. As I recall the results, clinostating did not have an adverse effect on the normal developmental pattern. The Soviets did that in relation to our Cosmos 782 project where they took some of the eggs that were exposed to spaceflight and exposed them to varying clinostat conditions during that same time period and as I recall they did not show an impact of the clinostat. Of course, the null gravity exposure of the eggs did not show an impact either in that system.

DR. ROUX: For what functions? The specific parameters that they studied were what?

DR. KEEFE: The eggs developed normally. As a matter of fact they developed better than ground-based organisms.

Bob?

DR. KRETSINGER: I was going to offer two sort of related observations. Roger in our session rather explicitly pointed out something I think we sort of were all intuitively aware of and that is the important distinction he made between the scaler component of gravity and what might be called directorial or directional component. If there is a standard reference direction that, for whatever reason an animal or plant depends on, it is highly improbable that a clinostat situation can rectify that. On the other hand, if one has reference to a scaler, if the mechanism is essentially scaler, then if it is time period, at least adequately long you should be able to average that out by tumbling it. I think that is a rather rigorous test.

DR. KEEFE: Do you want to get that one, Roger?

DR. TSIEN: It seemed to me in some systems the cells already have well-established axes like Stan's shoots have an axis and the way they react to gravity differs whether or not it is perpendicular or parallel to the shoot. Bones have long axes, and you put stresses on them or if we have blood pressure receptors between the top of our head and feet or if we sense the cardiovascular consequences. We have a long nexus, and in those only the component of the gravity parallel to that axis matters, whereas it may be in some of the dividing egg systems there is no axis to begin with, and it is not so much whether it is 1/2 G or 1 or 2 G's as long as it has a direction. My original notion was that perhaps for the scaler systems like the shoots zero gravity wasn't going to it be disruptive. The organism would have a means of adjusting to it because it has plenty of chances when it is already faced with zero component along the axis, such as when the shoot is already growing upright it has no reason to bend and so on.

So, I had guessed that maybe cells will know how to cope with zero G or organisms will know how to cope with that, whereas if they demand a direction and are never on earth without a direction except if they are rapidly tumbled then if they are, whatever follows from gravity sensing cannot follow the tumbling. Now, if it is a rapidly sensing thing, then clinostating is not equivalent to zero G.

DR. WATTERSON: I would like to address a point to that. We talked around this in our group, too: i.e., methods and systems to use to study different force fields. I tend to think in terms of force, whether it is a chemical signal, electrical or what, and what systems do people and can people look at: multiple force fields, gravity, whether you are using a clinostat or hypergravity, hypogravity, electrical fields, magnetic fields, etc. Are there phenomenologies that you can look at in multiple kinds of force fields to see what results? Do you find similar things occurring in, let us say, hypergravity as in a magnetic field? Do people study that, and can you do that?

DR. TSIEN: Perhaps people should look at the magnetic sensing organisms. I mention that particularly because it is a weak mechanical stimulus which is more likely to be analogous in more stages than response to light, say, or electricity which is going to have a very different fundamental input sensing mechanism. For gravity what we need is to condense more buoyant objects in the middle, and in both cases the struggle is to my mind not with lever arms and mechanism because I can make a mechanism that works with no lever arms just because it sends out diffusable chemical transmitter, say, cyclic AMP and there is no mechanical lever necessary. It is just diffusion gradients, but what is more important is brownian motion. That is what sets the sensitivity thermal agitation, not whether you can imagine a lever arm because you don't even need one.

DR. ROSS: I would like to speak to that about the buoyancy thing. In my own thinking about otoconia, they don't have to move. All you have to have is the stress on them on the molecular configuration, and you are really talking about interchangeable forms of energy. So I don't know what you mean by motility or how you are approaching this. I mean I would like to have the answer to that, too, perhaps, just because I think about it in an entirely different way. All we have to do is have something that can sense some change, whatever it might be.

DR. KEEFE: My point with the otoconia was that we have, consciously, anyway, become dependent upon the otoconial system for our conscious perception of gravity which does not necessarily feed over to the individual cell, e.g., an intestinal epithelial cell responding to a gravity vector. We are not as aware, for instance, of the gravity impacting on our individual cells as perhaps a plant is.

One of my problems is why is a plant so much more sensitive to gravity and changes in gravity than an animal, and of course, some of it is elastic in terms of the animal body.

DR. ROSS: I have got my legs crossed here,and I would submit my leg is very sensitive to gravity, and it has nothing to do with my vestibular system.

DR. KEEFE: No, that is proprioception and that would be something else. That would be a second conscious perception system as opposed to a perception at the individual cellular level by a cell. For most cells I would argue it would not make a whit of difference. The force of gravity at the cellular level is so much less than binding forces and gel forces, basement membrane and so forth. So, it really would not impact there, but I get into a problem when I start considering development and the laying down of the initial pattern of those tissues and how they get all tied together into proper functioning relationships and before that anchoring into the tissue and organ relationships occurs, then potentially those are capable of responding to an altered gravitational force.

Now, you, also, argue that at the moment to take Roger's idea a little bit here, you are carrying a fetus around, that fetus is going random in terms of vector of gravity. It really is not being consistently steadily exposed to 1 G in one direction. So, again, we don't know whether or not that is the absolute case.

Bob?

DR. KRETSINGER: I was going to pursue two comments that both Marty and Roger made that there are actually a sort of variety of weak forces that have consistently surprised biologists by the ability of organisms to detect them. Perhaps some of you could tell me who have thought about it and done the calculation, more recently. I think inevitably in an analysis of these systems one ultimately comes down to considering thermalnoise as the limit of detection, as setting the limit of detection, and brownian motion is one representation of that. Some of the examples are really striking, and I think all of them essentially contradicted conventional wisdom at the time, and one is the infrared receptors of a certain snake. There is a very, very small amount of energy there, but it is quite adequately detected, and of course, the other is the magnetic sensing bacteria the magnatite crystals and two NRC examples that have come out of the work on homing pigeons are their baro-receptors that can distinguish a difference in altitude less than the height of this room. They can tell how high they are absolutely using baro-receptors. The forth one, the second to come from the pigeons is the ability to sense very, very low frequency sound waves. You know, such a pigeon here can pick up 2 or 3 hertz sound waves, and that is the sort of thing you get from waves tumbling and clouds going over mountains. A pigeon could sit here and hear the waves out at the beach 20 miles away, I mean really hear. All of these were surprising until one calculates through the thermal energy and found that yes, in fact, they were right above thermal energies.

There may or may not be a commonality in the molecular mechanisms involved, but it would strike me as something worth looking at to see what the formal limits are in detection of different sorts of energy and the potential transduction mechanisms that are formally allowed and disallowed.

I wonder if anyone is actually familiar with what we are talking about in gravity detection. Do we have three or four decades margin or are we really at the limit of thermal noise?

DR. KEEFE: I think the plant system is fairly well known. Am I wrong? This is a non-plant person talking, saying that the plant system is fairly well known.

And how sensitive is it in terms of range?

DR. ROUX: One G for two minutes, something like that?

DR. KRETSINGER: But what does that mean relative to thermal noise?

DR. TSIEN: I tried to do a calculation. One type of sensing mechanism is where you have got -- just for simplicity I will speak of it being heavier than the surrounding medium, something like the, what is the name of the starch granule?

DR. KEEFE: Amyloplasts.

DR. TSIEN: Amyloplasts. In the simplest case it has two possible positions, and naturally gravity wants to pull it down and thermal noise wants to randomize it. In zero gravity it will spend equal time in the two positions, and gravity will somewhat tend to favor the lower one. Well by how much? If the volume of this thing is V, and it has a delta specific gravity (SG) against its surrounding medium of that, then its effective math is V delta SG and then we have to multiply it by the acceleration of gravity when we have got 1 G and then we multiply it by height that it can go between the two, and we ask that that be greater than KP. All right. I get for that when I take G as 9.8, whatever, if I have done this correctly, V delta SG times H is equal to 0.4 cubic microns to the fourth power. So, if you have a 1 cubic micron lump which has a density difference of one in the surrounding medium, like it is a density 2 or 0, like if it is air filled, then it has to be able to move at least 0.4 microns, excursion between its limits or else thermal energy will effectively randomize it, and that is a question we can ask the ultra structure people. Do you see things like that that are big enough, and, also, if it is only one small bit of the total mass, the mass isn't rigid, then that only makes things worse because the effective mass and volume are smaller, in that there is more thermal noise messing it up. So you want the biggest possible object, and I was wondering if your cells, Stan, which are only, you said, 10 or 15 microns across and probably won't be a full unit of specific gravity difference, maybe right away from that you can tell it is either the whole cell inside a cell wall or the tonoplast inside the cell, but maybe there is nothing else big enough.

DR. JAFFE: Is this taking into account cytoplasmic streaming which may be a force acting at right angles to this?

DR. TSIEN: No, of course not, but I don't see how a force acting at right angles will matter. There is no back reaction force on this particle. It doesn't have to do any work. There is a magic sensor that knows where it is without putting any back force on it which is the ideal case, and any back force you put on it or work that this has to do will only make things worse in the detection.

Now, the other way you can do it, and I am not quite sure about how you make the physical condition between these is that you have a mass on a spring or sitting inside the box, and what you are looking at is not much of a displacement here but a compression and something that feels the force here and maybe this is attached to a channel and when you push it down you close the channel or maybe it is hanging from the top or something, and what I see here is the thing, again it is the mass, I mean you want V delta SG, if it is effective mass in the buoyant medium, and in zero gravity this will sit at some equilibrium position. Now, we put 1 G on and it will move. We have to get a spring constant here, and I would want to call it K, except that I already need K for the Boltzman constant. So, I will call it S for spring.

So, the equilibrium displacement due to gravity or the expectation of X is going to be where the weight of this thing just compressed the spring so you are back at equilibrium. So you have V delta SG is the effective mass now times gravity is the force on it. This is going to have to be S times the displacement, but what is the uncertainty in X? Well, thermal motion tells you that 1/2 KT is going to be 1/2 SX^2 or that is the expectation of the square, and what we would expect for signal-to-noise is that the average value of this displacement be more than the thermal wobble of this thing up and down on the spring. All right? I think that came out to be the mass has to be greater than the square root of SKT over G and there has got to be a delta SG somewhere here. Now, the funny thing is that this has got a different dependence on KT in this one. I don't know why that should be, and I fear I must have skipped something because there should be a smooth transition. You should be able to have a complete theory that will get you through the transition between these two limiting cases. What is the spring constant? That would seem to me to come on the side of skeleton, and people probably ought to know what the stiffness of microtubules, actin filaments is per length, and maybe the scientists will be able to sort of get an idea what S is, and the rest of course we know. So this would give us an idea whether it is detectable, and then you have to look for a mass that is big enough on the end of the spring.

DR. ROSS: I just wanted to say I would like to get together with you because otoconia are on three dimensional springs, but you have a system of these masses on proteins that are acting as three dimensional springs and the length of the spring is not the same between otoconia. The otoconia are all linked together. You are dealing with different kinds of masses and different positions, and I think it is a very complicated thing, but I would like to get together with you and see how you deal with it. I don't want to do it now; it takes too much time.

DR. TSIEN: The usual thing is you find out the force constant, the direction, put it into a matrix, diagonalize the matrix, and find the principal axes, Then those become uncoupled once you find the principal axes, and then you can ask what is the gravity component? It happens to have a component along each of the routes. I don't know in detail how to do it, but it is a standard problem.

DR. ROSS: It has not been done, I assure you.

DR. TSIEN: In principle they know how, but the physicist who knows how to do the calculations doesn't have your data. They just take what the actual shapes and elasticity are.

DR. ROUX: Thank you, Roger and Dick. Bob Cleland?

DR. CLELAND: My group consisted of Mark Jaffe, Mike Evans, Abe Krikorian, Chuck Caldwell and myself, and we touched on a number of questions as we went along. We talked a lot about auxins and the way it moves in plants, the way they might relate to calcium and to gravitropism. Are there indications that other plant hormones might in fact, interact with either calcium or with gravitropism or gravimorphogenetic responses, and what, if anything, is known about this. Like many of these things involving plants I think the frustrating part of it is just how little we actually know.

There is one very nice system indicating that other hormones can affect, can interact with calcium in plants, and this is the system that Joe Sanders and Peter Hepler have been working on, on the formation of buds in the moss, Funaria. Funaria grows out as a series of filaments. Under certain situations, it will produce offshoots off of these filaments which will then actually form buds. This can be induced by several agents, in particular by a hormone that is present in plants called cytokinin. Cytokinins are interesting hormones because they are involved to a certain extent in the control of cell division processes, at least in tissue cultures, callous situations. They are involved in the control of senescence. They are involved in a number of various developmental controls. They have the interesting property of being important in plants in the free form and yet they exist in nature as one of the bases in certain tRNA's in all organisms. We are totally lacking knowledge at this point as to whether this is just a happenstance or whether in fact there is a real connection between these two. But what has been shown here in this system is that when there is the formation of these buds in response to cytokinins there is an accumulation of calcium at the site on the filament where this outgrowth is going to take place. It would appear that one way or another the cytokinins are involved in directing where the calcium is going to appear and then the question is, is this cause and effect? I mean is the appearance of calcium in that particular spot along the filament, does that determine the fact that there is going to be an outgrowth there? They have recently done some very nice work using the A-23187 to show that in fact that the application of that to the filaments which will cause the ingress of calcium into these filaments will induce bud formation in the same way that the cytokinins do. This is, I think, clearly one of the most interesting and promising systems in plants for studying the interaction of calcium with any of the plant hormones.

We went on from there to talk about the possibility of calcium being involved in other hormones that might be involved in gravitropism. Again we have talked about the fact that auxin seems to be involved in gravitropism, but there is increasing evidence that, in fact, a second plant hormone, ethylene gas is, also, involved. A couple of years ago Frank Salisbury showed that if one adds inhibitors of the biosynthesis of ethylene or adds inhibitors of the action of ethylene to stems of large plants, that is not seedlings but fairly good-sized plants that this markedly reduces the ability of the stem to show any gravitropic effect. Mike Evans was telling us that the same thing is true with roots, that he can markedly reduce gravitropism in roots by inhibitors of ethylene biosynthesis. Is it just biosynthesis or, also, action?

DR. EVANS: We have only looked at biosynthesis.

DR. CLELAND: Only the biosynthesis, but this suggests to us that there has to be more careful examination of what the ethylene is doing in this system. Is it simply sort of the ultimate growth controlling agent in controlling the unequal growth as was originally suggested by Stan Berg or in fact, could it be involved in an earlier step in the actual graviperception process.

On the question of whether calcium is involved in ehtylene in any way, there is remarkably little evidence one way or another. There is some evidence to say that calcium is involved, is necessary for ethylene biosynthesis in senescent tissues, but there is, also, evidence to say that it is not involved perhaps in ethylene biosynthesis in stems, and so that is a matter that still remains open.

We talked a little bit about the need to try to dissect the gravitropic response into individual parts. For example, one way of doing this would be to make more use of the gravitropic response in roots where the perception occurs in the cap, but the actual response occurs a considerable distance away from the cap.

For example, following the example as Stan Roux has developed with coleoptiles where there appears to be an asymmetric distribution of calcium in the gravitropism, one of the questions that would be interesting to know is is that asymmetric distribution necessary or involved directly in the unequal growth or is it involved in the actual perception; in other words, for instance, in a root you get unequal distribution of calcium in the cap which is not where any growth occurs but where the graviperception occurs or do you get unequal distribution in the growing zone, and this is something that would be relatively simple to look at but has not been done.

Another way of approaching it would be to ask what happens when you add the phenothiazines to individual parts of a root. You have a certain amount of problem with diffusion of course, but still it is certainly possible to add them either to the cap and see whether it is affecting the perception or having a greater effect on perception as adding it to the growth zone and seeing whether it is affecting the things which occur there.

Yesterday it was suggested that the water hyacinth system where you have a cap that can be removed that is very large might be an excellent one to look at. We don't happen to know, any of us whether the water hyacinth root shows any graviperception. That would be the first thing that would have to be tested obviously, but it is something you know, the kind of system that would be very interesting to look at in greater detail.

We talked about other possible systems that would be useful to study. We talked quite a bit about the use of lower plant systems, in particular the _Caulerpa_ system which Bill Jacobs has studied which is an unusual one. _Caulerpa_ is a huge unicellular alga. It is coenocytic alga in that it supposedly has no crosswalls but it does have as Abe Krikorian was pointing out some sort of tubule system through it that at least partly divides it up into compartments. As it grows it grows horizontally; it produces blades on the top and rhizoids on the bottom. You have the situation that if you turn this alga over after a short lag it will proceed to now reorient what it is producing so that what was the bottom side will now produce blades instead of rhizoids and vice versa. It is an interesting system and yet it has been one that has been very difficult to actually get down and test at any sort of biochemical level.

Another system that was raised as a possibility is the growing stem of a California poppy. As it starts to grow, the flower stalk is negatively geotropic, that is, it grows up. Then when the bud reaches a certain stage it proceeds to grow down, turn over and become positively geotropic. At a later stage it now goes up again and becomes negatively geotropic. This is the kind of system that might be very interesting to look at to see what kinds of things were going on here.

Finally we talked a little bit about the necessity for further examination of what goes on in the growth of plants in space. The results of the recent space experiments of Alan Brown and Joe Cowles indicate that the growth of the plants in the shuttle was not nearly as normal as people anticipated and that particularly there were seen to be real abnormalities in the direction of growth of the roots. This suggests to us that, to an even greater extent than we believed before, examination over a longer period of time in shuttle experiments is certainly going to be worthwhile.

Is there anything else that others want to add to that?

DR. ROUX: Analysis of calcium distribution in roots being done right now. We already have some preliminary data.

Thank you, Bob.

Bob Bandurski?

DR. BANDURSKI: I think that checkout time and other commitments may limit my presentation, but the discussion seemed to center around biorhythms for one, whether or not these would be set or would not be set in essentially a zero environmental stimulus input situation. We, also, tried to concentrate on things that could be measured by remote sensing devices and relatively easily monitored and again, bioluminescence appears to be one of the very, very strong ones.

My group consisted, by the way, of Milt Cormier and Barbara Pickard and Peter Reed, Ken Robinson, Stan Roux, Thora Halstead and myself.

And Dr. Cormier, again, stressed the importance of Rinella and the fact that it has a calcium metabolism response. It could be very easily monitored, because the fluorescence could be monitored by remote sensing instrument. There are other organisms that we hope we could grow in space that would have zero G all of its life, but apparently these systems are not that well understood.

By the way I think Dr. Zielinski yesterday, again stressed that it really would be nice to have a thing that grew its entire lifetime at zero G and then ask how it copes with life when it comes back. This is the kind of thing which we could gloss over, but I think it should really be looked at.

So one of the questions you could monitor with the jellyfish or the Rinella is whether or not calcium metabolism is upset by zero G and that is doable.

There was, also, some discussion, particularly by Dr. Pickard about the dinoflagellates which again show an endogenous rhythm and again could be monitored. They, too have very intricate exoskeletons and perturbations of that exoskeleton could be looked at.

Then an almost totally unrelated thing, but we began talking about are there other kinds of ionophores? Can people tailor ionophores with the right configuration so we can make those specific for manganese or specific for magnesium or specific for zinc. The probability is that right now nobody has the ability to make truly specific ionophores, but there would be hopes. After all, nature in her infinite wisdom has created proteins that have absolute specificity for ionic compounds and so I think in that direction ultimately one might be able to make something. Whether or not you could get them into membranes and so on would be another problem.

Then another question we talked about is whether we could do anything to counteract things like decalcification of bone, whether anything could be done by restricting the amount of movement into the plasma or secretion, whether that could be handled. Apparently no readily doable way at the moment.

Lastly, I think Dr. Robinson spoke about his *Fucus* eggs that we don't know really how they would orientate themselves if they had no environmental input or essentially none, and that, too, would be an important sort of thing. Now, it might simply be that the point of sperm entry would then become the stimulus and might become the orientating thing, but at least one could find out whether it would be random at that point or whether it would always be at the point of sperm entry.

I think that is about it. I would like to ask my group to make their comments, but that is about all I recall from the session. I guess the emphasis would be upon easily monitored things having to do with bioluminescence or with biorhythms or with both.

DR. TSIEN: You mentioned very briefly that there was no treatment you could think of to prevent the bone loss. I was just wondering, isn't there some way of playing with the parathyroid, vitamin D3, calcitonin? On ground you can make bone grow abnormally. You can emphasize calcium bone formation from the blood by feeding the right mix of hormones, can't you, or do you have to ablate the pituitary, the parathyroid? I have forgotten which hormone works.

DR. HALSTEAD: I think one of the most frustrating things about working with bone is it is much easier to affect the resorbtion side of bone than the formation side. And most of the hormones that we currently know of do affect resorbtion either by shutting it off or turning it on.

DR. TSIEN: It would not be ideal to work on the resorbtion side, but suppose you did inhibit resorbtion, so that deposition wouldn't be perfectly normal, but at least you would not have the net gross change. Has anyone tried that?

DR. BIKLE: There are a number of fairly important avenues of bone resorbtion. To answer your question quickly, nobody has done that in terms of trying to reduce the effects of gravity on bone. Yes, we do have access to materials like diaphosphonase which does inhibit bone resorbtion. We have estrogens which seem to block the effect of parathyroid induced bone resorbtion. We have agents that can work to stimulate bone formation such as fluoride, and the presence of adequate amounts of calcium stimulates bone formation. There are some reasonably good data to suggest that one of the vitamin D metabolites namely 24, 25, dihydroxy vitamin D probably stimulates bone formation. So those experiments are ripe to be done. They have not been done.

DR. TSIEN: Because if you could mill it out, then it would not only be useful; it would, also, eliminate a large class of mechanisms, if you could match the profile or defeat the profile by administering generalized systemic hormone.

DR. BIKLE: One of the complicating factors is that bone resorbtion appears to be a local or a bone-to-bone effect and that if you unweight one bone you don't get the generalized systemic effect. So there is some concern about whether the use of systemic type of modulators of bone resorbtion and bone formation is taking a sledgehammer to crack a walnut. That is a bit of a slowdown. It may be a cop-out, and it may be that they will, in fact, work and work differentially on bones in which there is a local stimulation to bone resorption.

DR. TSIEN: But at the moment the walnut remains uncracked. So maybe the sledgehammer --

DR. BIKLE: I don't dispute that.

DR. BANDURSKI: I think that is a very good comment because we had approached that from the point of whether or not a channel-making ionophore could be made that could prevent the loss, and since that cannot be done, maybe you are quite right. Maybe an alternative hormonal treatment might accomplish the same thing.

DR. KEEFE: I have a comment, not a question. Not to be contentious, but I think it is a bit misleading to refer to an absolute affinity for an ion. I mean almost inevitably one sees several decades difference in affinity, and sometimes those decades are enough that effectively it is absolute, but nearly any of these things that are advertised being specific for calcium have several decades specificity relative to other divalent and trivalents.

DR. BANDURSKI: Yes, that is absolutely correct.

DR. KEEFE: It may be a picayune point.

DR. BANDURSKI: No. And in classical enzymology you know, usually there is maybe a two order of magnitude difference but that is usually about all one sees. That is quite correct.

You had a comment?

DR. WATTERSON: It was the same thing, the proteins on the absolute specificity. Also, the environment they are in, where they are in the system makes a difference.

DR. BANDURSKI: I should not have said absolute. I should have said a couple of orders of magnitude, I guess.

DR. ROUX: Thank you, Bob.

Okay, and finally, Dan Bikle.

DR. BIKLE: I, also, feel the press of time, and will probably not do justice to a lot of very good, very stimulating discussion that came out of our group which was composed of Drs. Marty Watterson, Jim Chafouleas, George Malacinski and Muriel Ross and Lisette Lagace.

Now, we addressed two questions. The first question was how do you study the effects of gravity here on earth and in particular what are the models that we should be looking at now. We were not involved with plants in our group, but we were looking at models in general. The second question that we dealt with was what are the intracellular transducing mechanisms by which animals sense gravity and what were those signals.

Okay, first question, we came up with a number of different models which we explored in greater and lesser depth. I am not going to deal with ones that have been covered, in the interests of time, and point out some ones that have not been mentioned so far.

Rasmussen pointed out to us work by an individual named Daniel Alkon who has been working with an organism called Hermissenda. This particular organism has the advantage of having a photo receptor linked to gravitational receptor, in that you can train this particular microorganism to respond to light.

DR. BENNETT: It is a little mollusk.

DR. BIKLE: Little mollusk. Apparently you can modulate that trained response to light by modulating the gravitational field. Apparently there is a link between the gravitational receptor and the photo receptor which is mediated by a calcium regulated protein kinase. Has that got the gist?

DR. BENNETT: I think so.

DR. BIKLE: All right. So there is one model that has not been talked about before that might be potentially useful. We talked about Dictyostelium and the fact that during the process of the fruiting, the formation of the fruiting body there was a change in the modulation of the actin, the genes for actin that got turned on and off during that process changed. The question is will that be a gravitational response and if so what kind of signals would mediate that. The third model that we talked about in some length because of Dr. Malacinski's interest is the amphibian egg which is a polarized system, clearly affected by gravity. The question is what is in the egg which causes the sense of gravity?

The particular question that we raised which starts to get us into our second question concerns the cytoskeleton within the amphibian egg. What does that have to do with the sensing of gravity? We developed a number of types of experiments that would be easy to do in hypogravity. As a very basic starter one could fly these eggs and find out first of all what the effects are of the hyper G which occurs during takeoff and subsequently is there a memory associated with that in terms of the development of the egg? What happens to the egg in flight during hypo G, in terms of development? We could look at developmental features but we could, also, do transmission electron microscopy to look at something like the cytoskeleton inside the egg, as well as the orientation of the various subcellular organelles in the egg.

Marty brought up Chlamydomonas and the calcium avoidance behavior which is demonstrated by that particular organism and also the phenomenon of regeneration of flagella once you clip them off. How would that be affected by gravity, since that is clearly involved in the means of motility of this particular organism. The big point that was stressed in my group which I think comes about because of our lack of success yesterday in trying to deal with something like bone itself is that right now we need simple models by which to study the effects of gravity, that we should not try to concentrate too much of our efforts on a complex multicellular system but to develop the simple ones that can be studied readily here on earth and then flown in space.

The second question concerned the transducers that might exist inside the cell. We spent most of our time dealing with cytoskeleton and in particular we discussed two components of the cytoskeleton, the microtubules and then the microfilamentous network that is inside the cell. I have been impressed by work done by Keith Porter in developing the concept of microtrabeculae inside the cell from which these various organelles inside the cell are suspended and how they tie in to the shape-forming structural component of the microtubules.

We know that both pressure, and you could translate that into hyper G if you like, as well as cold causes a depolymerization of at least some of the microtubules. Jim Chafouleas pointed out to us that those microtubules which responded to pressure turned out to be the ones which appear to be regulated by calmodulin and calcium vis-a-vis the ability of calmodulin to sensitize those microtubules to depolymerization in the presence of calcium.

The question about hypo G is that, does that translate into some sort of gravitational receptor potential? That needs to be looked at and might in fact prove very meaningful. We don't know very much about that microfilamentous structure in terms of whether that in fact, would be changed by pressure, or the flip side of that, whether that would be changed in situations of hypo G. This is clearly a real possibility and if it turns out that microfilamentous structure or microtrabeculae would be influenced by gravity, then the next obvious thing to look at is the effect of calcium/calmodulin mediated mechanisms by which some sort of depolymerization, repolymerization could take place.

I think I will stop there. There are lots of other points. We started to get into the otoconia and the types of transducing mechanisms that might be involved with otoconia, but we really ran out of time before we got too far except that I guess I should point out that Muriel wants to emphasize the fact that we need not be dealing with a mechanical receptor type mechanism with otoconia but in fact the transduction mechanism there might be a change in electrical field which is generated by the piezoelectric forces caused by deformation of the otoconia in the presence of a gravitational field. So, the otoconia give us a good model for looking at a number of different possibilities for transduction inside the cell, but we did not have enough time to explore that further.

DR. WATTERSON: The main point was we could not come up with any suggested model systems for biomineralization, a simple system that could be looked at mechanically without man's intervention or studies, that kind of thing.

DR. BIKLE: Right.

DR. ROSS: I think it is important to stress that we felt that what we should be looking at is biomineralization and the problems that any cell faces in making something that is mineralized and that we could all, no matter what particular thing we are studying, learn something.

DR. BIKLE: I did not mention that at all, but that is correct.

DR. HEPLER: I don't have the exact figures. Perhaps Roger could help us out here, but I would imagine that the pressures required to depolymerize microtubules are way greater than you would get in any gravitational field.

DR. TSIEN: I emphasize a further problem. You don't have to use microtubules. There is a protein, for example, from the protozoan, called spasmoneme (JCB 77:358, 1978). It has a very remarkable property. You put calcium on it, and it shrinks like a rubber band shrinks, and I believe that it is reversible. You pull on it, and it will push calcium out. It happens to be in only a few protozoa, but why couldn't we have that inside the cell? And the problem with that is that is okay for an auditory hair cell which is looking in AC and then you rapidly release and take up calcium in an auscultatory way. The problem is that for a static system like a gravity responding one any fixed buffer inside the cell cannot keep acting for very long. You stretch it, say. Suppose you stretch it and it tries to release calcium? In a minute or two it will have released all its calcium and then it will be no use to the rest of the organism. Now, if it had cyclic AMP you could have because you can generate cyclic AMP indefinitely out of ATP and it seems to appear from nowhere, but calcium you cannot generate out of nothing. You have to generate from the store, and the store is always exhaustible rapidly. Anytime you put a buffer in a cell, and Dr. Rasmussen emphasized this as well, its effects are not going to last that long, and so I would doubt that first the microfilament pressures are wrong, but if you do -- you could find protein which would release calcium when you stretched it or pushed it, but as long as it stays entirely intracellular, I don't think it will do you any good for more than one minute, and in order to get a maintained calcium gradient you have to tap the plasma membrane with a channel or pump.

DR. CHAFOULEAS: I think as far as the cytoskeletal concerns, we were asking very primal questions. What happens to it when it goes up in the air? It is at zero G and etc., and so we were just asking are there changes in the organization.

DR. BIKLE: Yes, let me amplify that we all appreciate the fact that we are interested not so much in what high pressure does to microtubules. We appreciate that those are certainly not likely to be physiologic under any circumstances, but we don't know what happens on the flip side when we go down to zero G in terms of the microtubular structure, and that is the point I want to get. I am taking the pressure as merely something that we know something about. Microtubules clearly respond to high pressures. That is not a physiologic response. We don't know what would happen to the microtubules under conditions of low pressure, and there is reason to think that microtubules evolved under circumstances to maintain cell shape in the presence of forces which cause it to round up and we don't know what would happen when those forces are no longer extant.

DR. TSIEN: Pressure is different from tension on the microfilament. You see the pressure depolymerization is an actual direct effect on the microfilament and it needs a volume change in the microfilament in the sense that just as you have to have a mass to sense gravity, you have to have a volume change, and I very much doubt that absolute pressure would put tension on. That is a different physical stimulus to the microtubule than isotropic hydrostatic pressure, but then the second argument comes that even if the tension did do something to the calcium binding, calcium, at least, is a poor messenger to carry further effects.

DR. WATTERSON: I think we are getting into a bit of semantics. We discussed the spasmoneme example in the discussion group. The point is that there are obviously potential effects on cytoskeletal structures. The data are not there. We can speculate all we want to. What we were trying to come up with was straightforward experiments that can be done to get fundamental data that would allow us to make further postulation, further theoretical calculations. The data are not there. There are testable systems. I think Jim's point is well taken in terms of the fact that we just don't know what is going to happen when there is prolonged exposure to hypogravity and you return back again into a system where you have a force on it. Whether you want to define that in Newtonian terms or quantum mechanics or anything else is another whole story.

DR. ROSS: You just stressed, too, that what we talked about were things we could do mechanically so that we really were looking at the systems in a way that would require very little intervention.

DR. BIKLE: We did to some extent deal with that, although that wasn't an explicit topic. The electron microscopy, for example, fixing samples in space could be done mechanically and the types of experiments that we were thinking about flying were ones that were designed to be simple and mechanical, at least to get this basic first step in intervention. I, quite frankly think that to get good data we are going to require some body up there. That is my personal interjection.

DR. ROUX: Before bringing this session to a close I want to make one comment with respect to the discussion that just ended and that is those of you who are not familiar with the NASA program may have gotten the impression that all of the experiments or most of the experiments that are planned by NASA have to do with space shuttle experiments or experiments at hypo G. I should emphasize if you don't already know that the program that Dr. Halstead runs is primarily interested in earth experiments which will allow us to understand how gravity affects organisms, and most of the people in this room, as a matter of fact, are primarily concerned with experiments we can do here and now. So, as you disperse to the four winds, in thinking about some of the things that went on in this workshop, do not think only in terms of experiments that would have to be done at hypo G to get meaningful answers but try to think imaginatively of things that could be done here on earth which would comment on how gravity affects organisms. As a matter of fact, I would say most of Thora's program is focused on that. There will be more opportunities soon, we hope, for many more experiments in outer space. So, it is not unreasonable to be thinking in those terms as well, but certainly it would be inappropriate to restrict your thinking only to those kinds of experiments.

As you know, all of this material has been taped, and the tapes will be transcribed. I will receive a copy of the transcription and roughly edit it, and send that portion of the transcript which refers to your talk, those of you who gave talks, to you just for final look at it. If you want to make any changes, you can make changes at that time, and send it back to us. This is not a publication. This is not going to be a reference for anybody but this is for information.

I want to then finish up by expressing my deep appreciation to all of you for your cooperation in this really excellent workshop. I think I have gotten some insights into a lot of different things that are going on, and I really appreciate your input into this. I have heard many of you make comments about the really excellent quality of the talks and discussion. I think we owe a very special debt of thanks to Dr. Thora Halstead who had the original idea for this workshop. It came from her. I kind of put the things in motion but without her getting the idea initially, it never would have happened. So, a special thanks to you, Thora, for this. It was a very special privilege for me to cooperate with you on this workshop.

Also, a special thanks to Pat Russell who helped me in coordinating everything and getting your signatures and a lot of other things that I probably don't even know about. And Bob Krauss isn't here, but thanks, Bob, wherever you are, because obviously he was our main host for the facility that we have here.

I appreciate all of your inputs, and I will be in contact with you in letters and on phone conversations to follow up with some of the ideas originated here. Please, if you have any further desire to learn more about the NASA program or to participate in it and you have any questions about it, you can either direct those questions to me or to Thora.

Thank you very much.

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